Epidemiology of ESBL

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About pigs and humans

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(met een samenvatting in het Nederlands)

Proefschrift

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Chapter 1

General introduction

Background

Extended-spectrum beta-lactamases (ESBL)-producing Enterobacteriaceae are of great concern for public health. *Klebsiella pneumoniae* and *Escherichia coli* (*E. coli*) remain the major ESBL-producing organisms isolated worldwide, but these enzymes have also been identified in several other members of the Enterobacteriaceae family[1]. ESBL inactivate beta-lactam antimicrobials by hydrolysis and therefore cause resistance to different beta lactam antimicrobials, including third- and fourth generation cephalosporins[2]. The genes encoding for ESBL are often located on plasmids, which can be transferred between different bacterial species. These aspects challenge the understanding of ESBL dynamics and therefore the control of transmission of ESBL. Also, coexistence with other types of antimicrobial resistance occurs. Many different ESBL genes exist, the most common ESBL genes belong to the TEM, SHV, and CTX-M families[3]. CTX-M type ESBL are the most predominant in humans. More specific, $bla_{\text{CTX-M-15}}$ is the most common type reported in humans globally followed by $bla_{\text{CTX-M-14}}$[4]. ESBL prevalence in humans vary depending on region of the world. In the Netherlands overall prevalence of ESBL-producing Enterobacteriaceae is reported to be 5%[5]. Gut colonization with ESBL is associated with clinical manifestations and attributes to the dissemination of ESBL genes in addition[6-9]. Clinical infections with ESBL is associated with prolonged hospital stays, increased morbidity, mortality, and health care costs[10,11].

Epidemiology and dynamics of ESBL is complex. Hospitalization, antimicrobial use and international travel are known risk factors for human ESBL carriage in humans[12-15]. There are many potential reservoirs for ESBL of significance for humans to become an ESBL carrier. Livestock can also serve as a reservoir for ESBL-producing Enterobacteriaceae and ESBL genes[4]. Besides ESBL in livestock, ESBL-producing Enterobacteriaceae have been reported in companion animals and wildlife[16-18]. Animal derived food products, mostly meat, and other food products such as vegetables can be contaminated with ESBL as well[19-22]. Another potential reservoir is the environment, ESBL have been detected in recreational water and sewage[23,24].

The variety of potential ESBL transmission routes makes it complex to attribute the role of direct contact with livestock as an occupational risk for ESBL carriage. High use of antimicrobials and inappropriate use of cephalosporins in livestock production are associated with the emergence and high prevalence of ESBL-producing Enterobacteriaceae in animals[25,26]. A variety of ESBL have been identified in Enterobacteriaceae derived from food-producing animals worldwide. The occurrence of different ESBL types depends on animal species and geographical area. In Europe,

bla$_{CTX-M-1}$ is common in poultry, cattle and pigs, but in Asia mainly *bla*$_{CTX-M-14}$ is reported in poultry and other types of the CTX-M family (*bla*$_{CTX-M-2}$, *bla*$_{CTX-M-14}$ and *bla*$_{CTX-M-15}$) are reported in cattle and pigs[4].

Since around 2000, ESBL-producing Enterobacteriaceae have emerged globally, in both humans and animals[4]. An increasing interest was raised for the transmission of ESBL from animal to human. This focus was enhanced by publication of data where a certain level of molecular relatedness was found between human clinical isolates and isolates obtained from poultry and chicken retail meat[27]. A degree of similarity in ESBL genes, plasmids and *E. coli* genotypes found in humans and livestock is suggestive for transmission. Results on ESBL and other antimicrobial resistant bacteria suggest that transmission from animals to humans can occur through (in) direct contact with livestock or animal derived food products during work[28-30]. Next to the potential occupational health risk for farmers and slaughterhouse workers, further efflux of ESBL from (humans exposed to) livestock or food products into the general population might occur. In the Netherlands, most information on ESBL in animals and the potential transmission to humans was obtained in the poultry field. An overview on the distribution of ESBL genes in pigs, pig farming community and slaughterhouse workers in the Netherlands was not available yet. Therefore, sufficient data to quantify the relevant importance of this route of transmission was not available. Identification of ESBL-producing Enterobacteriaceae, ESBL genes and plasmids in humans and their exposure to ESBL on farms and in a slaughterhouse should provide further insights regarding personal and public health risks.

Objectives and outline

Considering the adverse effects that ESBL-producing Enterobacteriaceae from animals can have on public health and the gap in data on ESBL in pig farming, the main objective of this thesis is to assess the importance and relevance of the pig production chain for ESBL carriage in humans. A strong emphasis will be drawn to the occupational risk of acquiring ESBL when working with pigs or pig products. This main objective can be explained in several sub objectives, namely:

- To determine the prevalence of carriage of ESBL-producing *E. coli* in pig farmers, family members and employees;
- To assess human exposure to ESBL on pig farms via direct contact with pigs by determining the prevalence of ESBL-producing *E. coli* in pigs;
- To determine the association between ESBL carriage in humans living and/or working on pig farms and the presence of ESBL in pigs;

- To assess human exposure to ESBL on pig farms through air by determining the presence of ESBL in dust on pig farms;
- To determine the association between ESBL carriage in humans living and/or working on pig farms and the presence of ESBL in air;
- To determine the effect of the reduction of antimicrobial use and other farm management practices on the presence of ESBL-producing *E. coli* on pig farms;
- To determine the genetic similarity of ESBL-producing *E. coli* in pigs and humans within and between pig farms over time;
- To determine the prevalence of ESBL carriage in pig slaughterhouse workers;
- To determine the association between ESBL carriage in pig slaughterhouse workers and occupational exposure.

By collecting longitudinal data on ESBL in humans, pigs and the environment on pig farms and cross-sectional data in pig slaughterhouse workers, we aimed to gain more insight in the dynamics and complex epidemiology of ESBL in different human and livestock related reservoirs. In order to explore the importance for human health, the genetic characteristics of ESBL-producing *E. coli* are determined in both humans and animals to detect diversity and potential transfer of ESBL-producing *E. coli* and/or ESBL genes from animals to humans and vice versa.

All data regarding pig farming was collected by using the Bactopath study as sampling frame during the period 2011-2013. This study focused on the occurrence of livestock-associated Methicillin Resistant Staphylococcus Aureus (MRSA). MRSA carriage was known to be present on a large scale in pigs and pig farmers in the Netherlands. The Bactopath study aimed at identifying management measures (antimicrobial use, internal and external biosecurity, personal preventive measures) focusing on reducing MRSA exposure on pig farms to the benefit of pig farmers and public health. On 40 conventional pig farms in the Netherlands, samples from humans, animals and the environment were collected during four sampling moments with an interval of six months. For the purpose of identifying ESBL, rectal swabs were taken from 60 pigs from different age categories by a veterinarian. In addition, four electrostatic dust collectors (EDC's) were placed inside the stable and one EDC in the living house to collect dust during a two week period. All humans living and/or working on these pig farms were asked to provide a fresh feces sample. Questionnaires were used to assess personal and farm characteristics.

All data regarding pig slaughterhouse workers was collected in collaboration with the EFFORT (Ecology from Farm to Fork Of microbial drug Resistance and Transmission) project, a pan European study that focused (amongst other) on the resistome in the pig chain. Fecal swabs from slaughterhouse workers with occupational contact to live pigs, carcasses or food products were collected in June 2016.

In addition, our aim was to focus on the veal calve farming community as well. As part of ongoing research on 51 veal calve farms, 56 veal calve farmers and family members belonging to 22 farms provided a fecal sample. The presence of ESBL was determined phenotypically (prevalence of 9%). No samples from veal calves were available. Due to the low participation rate, further research was not pursued.

Cross-sectional results from the first sampling round of Bactopath were used to describe the prevalence of ESBL in farmers, family members, employees and pigs in chapter 2. In addition, the association between human ESBL carriage and presence of ESBL in pigs was analyzed as well. The possibility of ESBL transmission from animals to humans through air on pig farms was investigated in chapter 3. To gain more insight in human exposure to ESBL on pig farms and the potential of reducing this exposure, longitudinal animal data was analyzed for risk factors in chapter 4. Chapter 5 describes the presence and genetic characteristics of ESBL-producing *E. coli* in humans and pigs longitudinally within the same sample of pig farms and the association between them. The presence of ESBL in pig slaughterhouse workers and the association with their occupational exposure was determined in Chapter 6. Finally, overall results and the complexity of ESBL epidemiology were discussed in Chapter 7.

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Chapter 2

Carriage of extended-spectrum β-lactamases in pig farmers is associated with occurrence in pigs

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Abstract

Livestock may serve as a reservoir for extended-spectrum β-lactamase-producing Enterobacteriaceae (ESBL-PE). The objectives of this study were to determine the prevalence of carriage with ESBL-PE in pig farmers, family members, and employees, and its association with carriage in pigs. Rectal swabs were taken from 2388 pigs (398 pooled samples) on 40 pig farms and faecal samples were obtained from 142 humans living or working on 34 of these farms. Presence of ESBL-PE was determined by selective plating (agar). ESBL genes were analysed by PCR or microarray analysis, and gene sequencing. Genotypes and plasmids were determined by multilocus sequence typing and PCR-based replicon typing for selected isolates. ESBL genes were detected in *Escherichia coli* from eight humans (6%) ($bla_{CTX\text{-}M\text{-}1}$, n=6; $bla_{TEM\text{-}52}$, n=1 and $bla_{CTX\text{-}M\text{-}14}$, n=1) on six farms. In 157 pig isolates (107 pooled samples) on 18 farms (45%) ESBL genes were detected ($bla_{CTX\text{-}M\text{-}1}$, n=12; $bla_{TEM\text{-}52}$, n=6; and $bla_{CTX\text{-}M\text{-}14}$, n=3). Human and pig isolates within the same farm harboured similar ESBL gene types and had identical sequence and plasmid types on two farms (e.g. *E. coli* ST-453, $bla_{CTX\text{-}M\text{-}1}$, IncI1), suggesting clonal transmission. For the remaining farms, sequence types (ST), but not plasmid types, differed. Human ESBL carriage was associated with average number of hours working on the farm per week (OR=1.04, 95% CI=1.02-1.06) and presence of ESBLs in pigs (OR=12.5, 95% CI=1.4-111.7). Daily exposure to pigs carrying ESBL-PE is associated with ESBL carriage in humans.

Introduction

In Europe, an elevated prevalence of $bla_{CTX\text{-}M\text{-}1}$ has been reported in poultry, cattle, and pigs[1]. ESBLs have been detected in meat samples and transmission of ESBLs from livestock to humans through the food chain has been suggested[2-5]. Antimicrobial resistant Enterobacteriaceae can also be transmitted from live animals to humans[6], but evidence for a role of direct contact with livestock in human ESBL carriage is limited. Transmission of $bla_{CTX\text{-}M\text{-}1}$ harbouring IncI1 plasmids between pigs and farmers was suggested in a molecular study on two pig farms[7]. In another study, ESBL genes were detected in broiler isolates from all 26 farms studied and in isolates from six of 18 broiler farmers. Genetic similarities in genes and plasmids among isolates from farmers and broilers were documented on two farms[8]. ESBL carriage in farmers in that study (33%) is high compared to hospital patients in the southern part of the Netherlands (approximately 5%)[3]. It is also high when compared to ESBL carriage in people living in areas with high (3.6%) or low broiler densities (6.7%) in the Netherlands[9].

Direct contact with livestock may be an important risk factor for human ESBL carriage. The objectives of this study were to determine prevalence of carriage with ESBL-producing Enterobacteriaceae (ESBL-PE) in pig farmers, family members, and employees, and to determine the association between ESBL carriage in humans and pigs.

Materials and Methods

Study design

This is a cross-sectional analysis of samples that were derived in a longitudinal study. On 40 multiplier pig farms (sows and piglets present), with or without finishing pigs, faecal samples from farmers, family members and employees were collected using faeces cups (Minigrip®) and sent to the laboratory by mail between March and October 2011. On each farm, rectal samples from 60 pigs were collected by the farm veterinarian, using sterile cotton-wool swabs (Cultiplast®) and sent refrigerated to the laboratory by courier. All animal age groups present were sampled (sows, gilts, suckling piglets, weaning piglets and finishing pigs). Rectal swabs were combined in ten pools of six pigs. Each pool consisted of an age group in the same pen. Participants filled out questionnaires on general characteristics, farm activities, and intensity and duration of animal contact. Questionnaires on farm characteristics were filled out by veterinarians and farmers. The Medical Ethical Committee of the University Medical Centre Utrecht approved the study protocol (no. 10-471/K). All participants gave written informed consent.

Laboratory analysis

Pooled swabs from pigs and faecal samples from humans were analysed for the presence of ESBL-PE by selective plating. Samples were suspended in 10 ml peptone water and incubated overnight at 37°C. For screening of ESBL-PE, suspensions were cultured on selective agar plates (*Brilliance*™ ESBL Agar, Oxoid®) and incubated overnight at 37°C. When no growth was seen, plates were incubated another night at 37°C. Morphologically different colonies suspected of ESBL production were cultured individually on a blood agar plate (Oxoid®) and incubated overnight at 37°C. In case of morphological uncertainty an oxidase test was performed before culturing. Bacterial species identification of the isolates was performed by MALDI/TOF (Bruker®). For phenotypical confirmation of ESBL-PE, a 0.5 McFarland suspension was inoculated on a Mueller-Hinton agar and a combination disc test (ROSCO®) including cefotaxime, cefotaxime+clavulanate, ceftazidime, ceftazidime+clavulanate, cefepime, and cefepime+clavulanate (*Neo-Sensitabs*™) was used to confirm the presence of ESBL-PE (EUCAST guidelines, 2012). Isolates were stored at -80°C before molecular analysis.

DNA was isolated using UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories, Inc.). A $bla_{CTX\text{-}M}$ group 1 specific PCR was used to detect presence of the most prevalent ESBL gene group[10]. DNA from $bla_{CTX\text{-}M}$ group 1 positive isolates was sequenced using the same primers to determine the $bla_{CTX\text{-}M}$ group 1 gene-type. Isolates with a negative $bla_{CTX\text{-}M}$ group 1 specific PCR result were analysed using ESBL microarray (Check-MDR CT101, Checkpoints, Wageningen) to detect bla_{TEM} group, bla_{SHV} group or another $bla_{CTX\text{-}M}$ group (2, 8, 9, 25). DNA from ESBL microarray positive isolates was sequenced with group-specific primers to determine the exact gene type[11,12]. DNA sequences were interpreted with Basic Local Alignment Search Tool (National Center for Biotechnology Information). For bla_{SHV} and bla_{TEM} groups, sequences were also aligned with non-ESBL types ($bla_{TEM\text{-}1}$, $bla_{SHV\text{-}1}$) to determine the exact gene-type based on single nucleotide polymorphisms.

Genotyping and plasmid characterization were performed for human and pig isolates with similar ESBL genes identified in the same farm. For each human isolate, one pig isolate from the same farm was selected. Genotypes and plasmid types were determined by multilocus sequence typing (MLST) (MLST databases an UoW, UK) and PCR-based replicon typing (PBRT) respectively[13]. The location of the ESBL genes on plasmids was tested by transformation. *Escherichia coli* cells (*ElectroMAX DH10B*™ cells, *Life Technologies*) were used as recipient and LB agar plates containing cefotaxime (1 µg/ml) as selective agent. Whole genome sequencing was available for eight human and pig isolates, plasmid types and sequence types (ST) were reconstructed from this data[14].

Data analysis

Farms were classified as ESBL-positive if an ESBL gene was detected in an isolate from at least one pooled pig sample. Statistical analyses were performed using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). The association between ESBL carriage in humans and pigs was calculated with logistic regression analysis (proc GENMOD) adjusted for clustering at farm level. Presence of ESBLs in pigs, average number of hours working per week, and potential confounders age, gender, and smoking were analysed univariately and selected for multivariate analysis when p-value was <0.2. Model selection was performed by a backward procedure. Model fit was checked with the QIC-statistic (Quasi-likelihood under the Independence model Criterion). The final model retained variables significant at $p \leq 0.05$.

Results

Participant characteristics

In total, 142 people (47 farmers, 76 family members, and 19 employees) living/working on 34 of the 40 farms (one to 11 persons per farm) participated (Table 1). The response rate was 68%. The main reason for non-participation was the objection to collect faecal samples.

Farm characteristics and pig isolates

On average, 470 sows were present per farm (range 110-1100). Finishing pigs were present on 27 of 40 farms. Farms without finishing pigs either supplied pigs for finishing pig farms (n=11) or supplied gilts for other multiplier farms (n=2). On 23 farms gilts were supplied by another farm, the other 17 farms had their own gilt supply (closed farms).

From 40 farms, 398 pooled samples (ten pooled samples per farm, six rectal swabs each; on two farms, only nine pools were analysed) taken from 2388 pigs were analysed. In total, 168 pig isolates (114 pooled samples) from 23 farms were phenotypically confirmed ESBL-PE (with combination disc test) (*E. coli* (n=160), *Citrobacter freundii* (n=4), *Enterobacter cloacae* (n=1), Salmonella sp. (n=1), Escherichia sp. (n=1), and *Escherichia fergusoni* (n=1)). ESBL genes were detected in 157 isolates (107 pooled samples) from 18 farms (45%) (*E. coli* (n=154), Salmonella sp. (n=1), Escherichia sp. (n=1), and *E. fergusoni* (n=1)). Eleven phenotypically confirmed ESBL-PE isolates did not harbour one of the tested ESBL genes (*E. coli* (n=6), *Citrobacter freundii* (n=4), *Enterobacter cloacae* (n=1)). $bla_{\text{CTX-M-1}}$ was detected most frequently (12 farms, 89 isolates (57%)), followed by $bla_{\text{TEM-52}}$ (6 farms, 33 isolates (21%)) and $bla_{\text{CTX-M-14}}$ (3 farms, 18 isolates (11%)) (Table 2). ESBL gene presence in pooled samples ranged from 16% in (rearing) gilts to 29% in sows, sucking piglets, and finishing pigs (Table 3).

Table 1. General characteristics of the participants of the study (n=142).

Human Characteristics	Frequency (%)
Gender	
Male	85 (60)
Female	57 (40)
Category	
Farmer	47 (33)
Family of farmer	76 (54)
Employee	19 (13)
Age (mean, range)	142 (36, range 6-79)
Age <18 years	30 (21)
Age 18-65 years	109 (77)
Age >65 years	3 (2)
Living on farm	
Yes	113 (80)
No	29 (20)
Average number of hours working on the farm per week	134 (mean 25, range 0-80)
0	42 (31)
1-20	27 (20)
≥20	65 (49)
Frequency of pig contact	129
Daily	64 (50)
Weekly	25 (19)
Monthly	9 (7)
Less than monthly	31 (24)
Smoking	141
Yes	13 (9)
No	128 (91)

Table 2. ESBL genes in 157 pig and 12 human isolates.

Pig isolates					Human isolates	
Farm	**Positive pooled samples (n)**	**ESBL type 1 (isolates (n))**	**ESBL type 2 (isolates (n))**	**ESBL type 3 (isolates (n))**	**Person**	**Gene type (isolates (n))**
1	9/10	$bla_{CTX-M-1}$ (16)[1]				
2	10/10	$bla_{CTX-M-1}$ (16)				
3	9/10	$bla_{CTX-M-1}$ (13)			3a	$bla_{CTX-M-1}$ (1)
4	5/10	$bla_{CTX-M-1}$ (7)[2]				
5	5/10	$bla_{CTX-M-1}$ (7)			5a	$bla_{CTX-M-1}$ (1)
					5b	$bla_{CTX-M-1}$ (1)
6	4/9	$bla_{CTX-M-1}$ (4)				
7	1/10	$bla_{CTX-M-1}$ (2)[3]				
8	8/10	$bla_{CTX-M-1}$ (11)	bla_{TEM-52} (2)			
9	8/10	$bla_{CTX-M-1}$ (10)	$bla_{CTX-M-32}$ (1)		9a	$bla_{CTX-M-1}$ (1)
					9b	$bla_{CTX-M-1}$ (1)
10	5/10	bla_{TEM-52} (9)				
11	1/10	bla_{TEM-52} (2)[4]				
12	1/10	bla_{TEM-52} (1)				
13	8/10	bla_{TEM-52} (13)	$bla_{CTX-M-1}$ (1)	bla_{TEM} group 3 (1)[5]	13a	bla_{TEM-52} (2)
14	9/10	bla_{TEM-52} (6)	$bla_{CTX-M-2/97}$ (4)	$bla_{CTX-M-1}$ (1)		
15	6/10	$bla_{CTX-M-14}$ (7)			15a	$bla_{CTX-M-14}$ (2)
16	1/10	$bla_{CTX-M-14}$ (1)				
17	8/10	$bla_{CTX-M-14}$ (10)	$bla_{CTX-M-1}$ (1)			
18	9/10	$bla_{CTX-M-15}$ (11)				
19	0/10				19a	$bla_{CTX-M-1}$ (3)

1 CTX-M-1 isolates were not tested for additional genes
2 Six isolates were *Escherichia coli* and one isolate was Escherichia sp.
3 One isolate was *E. coli* and one isolate was Salmonella sp..
4 One isolate was *E. coli* and one isolate was *Escherichia fergusoni*.
5 Further specification by sequence analysis was unsuccessful.

Table 3. ESBL genes in pooled samples from pigs in different age groups.

Age group	Pooled samples (n)	Pooled samples with ESBL genes detected (n and (%))
Sows	76	22 (29)
(Rearing) gilts	77	12 (16)
Suckling piglets[1]	78	23 (29)
Weaned piglets	107	29 (27)
Finishing pigs	52	15 (29)
Total	390[2]	101 (26)

[1] Suckling piglets = pooled sample contained rectal swabs from one mother sow and five of her suckling piglets.
[2] No age group was reported for eight pools in one farm. On two farms, only nine pools were analysed.

Human isolates

From 142 humans, 16 isolates from 12 people were phenotypically confirmed as ESBL-PE (*E. coli* (n=13), *C. freundii* (n=1), *E. cloacae* (n=1), and *Proteus vulgaris* (n=1)). ESBL genes were detected in 12 isolates (all *E. coli*) from eight people (6%). $bla_{CTX\text{-}M\text{-}1}$ was found in isolates from six participants, the other two participants carried $bla_{TEM\text{-}52}$ and $bla_{CTX\text{-}M\text{-}14}$ positive isolates, respectively. Five out six humans who carried $bla_{CTX\text{-}M\text{-}1}$ were living/working on a farm where $bla_{CTX\text{-}M\text{-}1}$ was exclusively (n=3) or predominantly (n=2) detected in pig isolates. The farmer who carried $bla_{TEM\text{-}52}$ worked on a farm where $bla_{TEM\text{-}52}$ was predominantly detected in pig isolates and the farmer who carried $bla_{CTX\text{-}M\text{-}14}$ worked on a farm where $bla_{CTX\text{-}M\text{-}14}$ was exclusively detected in pig isolates (Table 2).

Prevalence of human ESBL carriage was 6% (95% Confidence Interval (CI) 2-9%). The prevalence among 64 individuals with daily pig contact was 11% (95% CI=3-19%) and among 52 individuals living/working on a farm with ESBL-PE carrying pigs prevalence was 13% (95% CI=4-23%). Prevalence was highest among 26 individuals with daily pig contact on a farm where ESBL genes were detected in pigs; 27% (95% CI=10-44%).

MLST and plasmid analyses

Bacterial strain typing by MLST and plasmid replicon typing were performed on seven human isolates and seven pig isolates from five farms (Table 4). Three sets of human and pig isolates were identical in ST and plasmid type. For example in farm 5, two human and two pig isolates were identified as *E. coli* ST-453, harbouring $bla_{CTX\text{-}M\text{-}1}$ on a IncI1 plasmid. In the remaining four pairs, plasmid types but not STs were identical. In three pairs, transformation did not succeed in one isolate.

Association between ESBL carriage in humans and pigs

ESBL carriage was detected in seven of 52 humans living/working on farms where ESBL genes were detected in pigs and in one of 90 humans living/working on farms where ESBL genes were not detected in pigs (OR=13.9, 95% CI=1.7-115.0, p=0.01) (Table 4). When this analysis was restricted to isolates harbouring $bla_{CTX\text{-}M\text{-}1}$, the association remained (OR=17.9, 95% CI=1.9-168.2, p=0.01). As the prevalence is too low to perform separate analyses for each gene type, no distinction in ESBL gene types is made in further analyses. ESBL carriage in humans was associated with average number of hours working on the farm per week (OR=1.04, 95% CI=1.02-1.06, p=0.0001), and age (OR=1.05, 95% CI >1.00-1.09, p=0.01). All carriers (six farmers and two family members) reported to work at least 20 hours per week on the farm. All seven carriers that lived on a farm with ESBL-PE carrying pigs reported to have daily pig contact. No differences in prevalence could be estimated for frequency of pig

contact, living on farm, and being a farmer, family member or employee because there was little or no variation within the group of carriers. Results of univariate analyses are presented in Table 5.

Age, average number of hours working on the farm per week, and presence of ESBLs in pigs were selected for multivariate analysis. Human carriage of ESBLs was positively associated to average number of hours working on farm per week (OR=1.04, 95% CI=1.02-1.06, p=0.0008), and presence of ESBLs in pigs (OR=12.5, 95% CI=1.4-111.7, p=0.02). The final model is presented in Table 5.

Table 4. STs of *E. coli* isolates and plasmid characterization.

Farm	Origin	Gene type	*E. coli* ST	Plasmid type
3	Human	$bla_{CTX-M-1}$	ST-540*	IncI1
	Pig	$bla_{CTX-M-1}$	ST-165[SLV*,1,†]	IncI1
5	Human	$bla_{CTX-M-1}$	ST-453*	IncI1*
	Human	$bla_{CTX-M-1}$	ST-453*	IncI1*
	Pig	$bla_{CTX-M-1}$	ST-453*	IncI1
	Pig	$bla_{CTX-M-1}$	ST-453*	IncI1
9	Human	$bla_{CTX-M-1}$	ST-711*	IncI1*
	Human	$bla_{CTX-M-1}$	NT[2,†]	IncI1, NTR[3]
	Pig	$bla_{CTX-M-1}$	ST-3321*	IncI1*
	Pig	$bla_{CTX-M-1}$	ST-711	IncI1
13	Human	bla_{TEM-52}	ST-10	IncI1, NTR
	Pig	bla_{TEM-52}	NT[4,†]	IncI1
15	Human	$bla_{CTX-M-14}$	ST-3079	*Coll*E2
	Pig	$bla_{CTX-M-14}$	ST-744[SLV5,†]	*Coll*E2, NTR

* Data constructed from WGS data[1,4]; † Allele codes are available as supportive information.

[1] Single locus variant of ST-165.

[2] New type, not registered yet.

[3] No successful transformation.

[4] New type, not registered yet. Not similar to other new type found.

[5] Single locus variant of ST-744.

Table 5. Univariate and multivariate analyses for ESBL carriage in farmers, family members and employees.

	Univariate analysis			Multivariate analysis		
Determinant	**OR**	**CI**	**p**	**OR**	**CI**	**p**
Age	1.05	>1.00-1.09	0.01			
per 10 years	1.7	1.1-2.5	0.01			
Gender (male vs female)	2.4	0.6-10.1	0.23			
Smoking (yes vs no)	1.3	0.1-12.2	0.80			
Average number of hours working on farm per week	1.04	1.02-1.06	0.0001	1.04	1.02-1.06	0.0008
per 10 hours	1.5	1.2-1.8	0.0001	1.5	1.2-1.8	0.0008
Presence of ESBLs in pigs (yes vs no)	13.9	1.7-115.0	0.01	12.5	1.4-111.7	0.02

Discussion

Among people working/living on pig farms, frequent contact with pigs carrying ESBL-PE is associated with ESBL carriage. Working hours on the farm (indicating direct contact with pigs) and presence of ESBLs in pigs on the farm were associated with human ESBL carriage. $bla_{CTX\text{-}M\text{-}1}$ was found in six of eight humans and 12 of 18 farms.

The overall prevalence for human ESBL carriage was 6% (95% CI=2-9%), comparable to the reported prevalence of 5% in a hospital patient population in the Netherlands[3], and to ESBL carriage in people living in Dutch municipalities with high or low broiler densities (3.6 and 6.7% respectively)[9]. In the current study, the prevalence was 13% (95% CI=4-23%) among people living/working on a farm with ESBL-PE carrying pigs, and 27% (95% CI=10-44%) among humans with daily exposure to ESBL-PE carrying pigs. The latter is comparable to the prevalence among 18 Dutch broiler farmers (33%) working on farms where ESBL genes were detected in broilers[8].

ESBL gene types detected in humans corresponded to those exclusively or predominantly detected in pigs on the respective farms. These results are suggestive for transmission of ESBL genes from pigs to humans (or vice versa). The similarity in ST and plasmid type between human and pig isolates found on two farms strongly suggest the occurrence of clonal transmission. This is endorsed by whole genome sequencing results; on farm 5, only six single nucleotide polymorphisms were found between one farmer isolate and two related pig isolates[14]. Results from other studies suggest that spread of ESBL genes in *E. coli* between animals and farmers

predominantly results from horizontal dissemination of plasmids, rather than from transmission of bacterial strains[7,8].

High use of antimicrobials in livestock production is considered to be associated with a high prevalence of ESBL-PE in animals[15]. Antimicrobial use in pigs on these farms is comparable to the use in the Dutch pig farm population[16].

Faecal samples were collected through self-sampling and sent to the laboratory by mail. As *E. coli* is known to survive in faeces for a longer period without refrigeration, it is unlikely that this led to under-detection of ESBL-PE[17,18].

After molecular analysis, ESBL genes were determined in 169/184 (92%) of the phenotypically determined ESBL-PE (both human and pig isolates), which is in line with the positive predictive value of 93% for local ESBL confirmation found in a multi-centre evaluation study[19]. In the present study, most Enterobacteriaceae other than *E. coli* with ESBL phenotype did not harbour ESBL genes. In four of 15 isolates, presence of AmpC β-lactamases was confirmed with the ESBL microarray used (data not shown). Inducible chromosomal AmpC is often present in Enterobacteriaceae other than *E. coli* and *Klebsiella pneumonia* and the presence of chromosomal or plasmid mediated AmpC β-lactamases or other β-lactamases might partially explain the discrepancy[19-23]. AmpC β-lactamases were not further explored in this study. Isolates with PCR-confirmed $bla_{CTX\text{-}M}$ group 1 were not further analysed with the ESBL-microarray. This may have resulted in underreporting of other ESBL genes present in the same isolate. Yet, the likelihood of multiple different ESBL genes in a single isolate is considered to be low[4].

Hospitalization and foreign travel are known risk factors for human carriage of ESBLs[24,25]. None of the eight ESBL carriers had been hospitalized or had used antibiotics in the preceding 12 months. Five ESBL-carriers had been abroad in the preceding 12 months (France (n=3), Scotland (n=1), Turkey (n=1)), and traveling data were missing from three carriers. ESBL-PE have been reported widely in livestock and meat[1-5,26], but also in healthy and diseased companion animals[27,28], wildlife[29], vegetables[30], drinking water[31], and sewage[30]. Duration of human ESBL carriage can be prolonged[32,33], although determinants influencing duration of carriage are not well understood. In the current cross-sectional study, duration of ESBL carriage was not determined, but a clear association between human ESBL carriage and direct contact with ESBL-PE carrying pigs was seen. Together with the increasing occurrence of ESBLs in livestock worldwide, livestock farmers appear to have a higher risk of carrying ESBLs than members of the general population. In this study we did not observe human ESBL carriers without frequent exposure to pigs. More research is needed on duration and risk of ESBL carriage. Moreover, the attributable role of ESBL carriage in farmers for public health remains unclear until more knowledge is gained on the transmission of ESBLs from farmers into the community.

Conclusion

Carriage of ESBL in people working/living on pig farms is associated with frequent contact with pigs carrying ESBL-PE. In both humans and pigs, $bla_{CTX\text{-}M\text{-}1}$ was the most frequently detected ESBL gene and genetic similarities were seen in plasmid types and STs.

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UND en VARKENSSLAGERU

Chapter 3

Air exposure as a possible route for ESBL in pig farmers

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Abstract

Livestock can carry extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae, with $bla_{CTX\text{-}M\text{-}1}$ being most prevalent. ESBL carriage in farmers is associated with ESBL carriage in animals, with direct animal-human contact considered as the dominant route of transmission. However, inhalation of stable air might represent another route of transmission. We, therefore, quantified presence of $bla_{CTX\text{-}M}$ group 1 genes (CTX-M-gr1) in dust and the association with CTX-M-gr1 carriage in pig farmers, family members and employees. We included 131 people living and/or working on 32 conventional Dutch pig production farms (farmers, family members and employees) during two sampling moments over a 12-month interval. Human stool samples, rectal swabs from 60 pigs per farm, and 2-5 dust samples collected using an electrostatic dust collector (EDC) (as a proxy for presence of viable CTX-M-gr1 carrying bacteria in air) were obtained per farm. Presence of ESBL-producing *Escherichia coli (E. coli)* in stool samples and rectal swabs was determined by selective plating and CTX-M-gr1 was identified by PCR. Dust samples were analyzed directly by PCR for presence of CTX-M-gr1. Questionnaires were used to collect information on nature, intensity and duration of animal contact. Overall human prevalence of CTX-M-gr1 carriage was 3.6%. CTX-M-gr1 was detected in dust on 26% of the farms and in pigs on 35% of the farms, on at least one sampling moment. Human CTX-M-gr1 carriage and presence of CTX-M-gr1 in dust were associated univariately (OR=12.4, 95% CI=2.7-57.1). In multivariate analysis human CTX-M-gr1 carriage was associated with the number of working hours per week (OR=1.03, 95% CI=1.00-1.06), presence of CTX-M-gr1 carrying pigs on the farm (OR=7.4, 95% CI=1.1-49.7) and presence of CTX-M-gr1 in dust (OR=3.5, 95% CI=0.6-20.9). These results leave open the possibility of airborne CTX-M-gr1 transmission from animals to humans next to direct contact.

Introduction

Livestock can carry extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae, with CTX-M-1 ESBL as the most important enzyme representatives in pigs in Europe[1-5]. Transmission of ESBL genes from animals to humans has been shown on pig farms[5-8]. Moreover, ESBL carriage in farmers is associated with ESBL carriage in pigs on the same farm[7]. Direct contact with pigs has been assumed as the dominant route of transmission. However, ESBL has also been detected in dust and air within pig farms[2,6,9-11]. Two German studies detected ESBL-producing Enterobacteriaceae in air on six out of 35 and four out of seven farms respectively[2,11]. In the latter study, no ESBL-producing Enterobacteriaceae were found in dust, while another German study did detect ESBL-producing bacteria in 11% of 282 collected dust samples on 47 pig farms[9]. Also in Germany, cefotaxime resistant *Escherichia coli* (*E. coli*) were detected in 11% of 95 dust samples collected on 48 farms[10]. Considering the presence of ESBL genes in air or dust, inhalation of stable air might represent a second route of transmission. The role of ESBL in air (and dust) as a risk factor for human ESBL carriage and potential route of transmission has not been explored yet. This might be important because transmission through air will involve a different spectrum of potential preventive measures than those related to direct contact. In a longitudinal study, we investigated the presence of ESBL genes belonging to $bla_{\text{CTX-M}}$ group 1 (CTX-M-gr1) in dust (as a proxy for exposure through air) in pig farms and the association with CTX-M-gr1 carriage in pig farmers, family members and employees.

Materials and Methods

Study design

The design of the study has been described partially in previous studies[7,12,13]. In short, 40 conventional Dutch pig production farms were enrolled in the study between March and October 2011. At two sampling moments, over a 12-month interval, human stool samples were obtained from pig farmers, family members and employees using faeces cups (Minigrip®) and sent to the laboratory by mail. Dust samples were obtained from the stables and the family home by using electrostatic dust collectors (EDC's). EDC's consist of two sterilized electrostatic dust cloths in a polypropylene sampler, which passively capture airborne settled dust[14]. Four EDC's were placed at different locations in the stables out of reach from pigs and at least one meter above the ground (for example on a windowsill) and one EDC was placed in the house (usually on the highest cupboard in the living room or kitchen of the house). The EDC's were left in place for a period of

two weeks, this collection time was needed to gather a sufficient amount of dust for DNA extraction. Afterwards they were send to the laboratory by mail where they were stored at −80 °C until analysis. Rectal samples from 60 pigs were collected on each farm by the farm veterinarian, using sterile cotton-wool swabs (Cultiplast®) and sent refrigerated to the laboratory by courier. All animal age groups present were sampled (sows, gilts, suckling piglets, weaning piglets and finishing pigs). Rectal swabs were combined in ten pools of six pigs. Each pool consisted of an age group in the same pen. Participants filled out questionnaires on general characteristics, farm activities, intensity and duration of animal contact and other risk factors for ESBL carriage such as traveling, hospitalization and antimicrobial use. Questionnaires on farm characteristics were filled out by veterinarians and farmers. The Medical Ethical Committee of the University Medical Centre Utrecht approved the study protocol (No. 10-471/K). All participants gave written informed consent.

Laboratory analysis

Human and pig samples

All faecal samples from humans and pooled swabs from pigs were analysed for the presence of ESBL-producing Enterobacteriaceae by selective plating as described previously[7]. Samples were suspended in 10 ml buffered peptone water and incubated overnight at 37°C. For screening of ESBL-producing Enterobacteriaceae, suspensions were cultured on selective agar plates (*Brilliance*™ ESBL Agar, Oxoid®) and incubated overnight at 37°C aerobically. In absence of growth, plates were incubated another night at 37°C. Morphologically different colonies suspected of ESBL production were cultured individually on a blood agar plate (Oxoid®) and incubated overnight at 37°C. In case of morphological uncertainty an oxidase test was performed before culturing. Bacterial species identification of the isolates was performed by MALDI/TOF (Bruker®). For phenotypical confirmation of ESBL-producing Enterobacteriaceae, a 0.5 McFarland suspension was inoculated on a Mueller Hinton agar and a combination disc test (ROSCO®) including cefotaxime, cefotaxime+clavulanate, ceftazidime, ceftazidime+clavulanate, cefepime, and cefepime+clavulanate (Neo-Sensitabs™) was used (according to the guidelines of the manufacturer (http://www.rosco.dk)). Isolates were stored at -80°C before molecular analysis.

In all ESBL-suspected *E. coli* the presence of CTX-M-gr1 was identified by PCR. Due to logistic reasons, the molecular analysis was performed in two laboratories. In the first laboratory, DNA from human isolates from both sampling moments and pig isolates from the first sampling moment was isolated using UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories, Inc.). A CTX-M-gr1 specific PCR was used to detect presence of CTX-M-gr1. DNA from CTX-M-gr1 positive isolates from humans

was sequenced using the same primers to determine the presence of CTX-M-gr1[15]. In the second laboratory, DNA from pig isolates from the second sampling moment was isolated using DNeasy 96 Blood & Tissue Kit (Qiagen). Real-Time PCR (SybrGreen, Life Technologies) was used to detect presence of CTX-M-gr1. PCR was repeated for a part of the DNA samples using a conventional PCR (BioMix Red, Bioline), since the Real-Time PCR wasn't optimized fully unfortunately for all DNA samples.

Dust samples

Dust samples were analyzed by qPCR for presence and quantification of CTX-M-gr1. First, dust was removed by homogenizing an EDC with 10 ml of pyrogen-free water with 0,05% Tween20 in a stomacher bag (VWR StarBlender) for 10 min. After repeating the homogenizing process with 10 ml of sample suspension, the resulting 16 ml were stored at -80°C for at least one night. Samples were freeze dried for 2-4 days to remove the extraction liquid. Extracted dust was stored at -80°C until DNA extraction. Fourteen extraction blanks were included, these consisted of EDC that were not exposed to air.

Second, DNA was extracted from dust. DNA extraction was performed using the Macherey-Nagel NucleoSpin® 8 Plant II kit (http://www.mn-net.com). The standard protocol was slightly modified by performing an initial bead beating step using a dry weight of 40 mg where possible (with double the amount of PL1 and RNase A, and 500 mg glass beads with a diameter of 212-300 µm in a FastPrep FP120 Cell disrupter at a speed of 6.5 for 45 s). For samples with >20 mg of dust extracted, the wash step with buffer PW1 was repeated. Finally, a single DNA elution step was performed.

Third, presence and amount of CTX-M-gr1 was determined. A SybrGreen qPCR assay using published primers[16], at an annealing temperature of 57.5°C, by use of the CFX384 system (Biorad) was performed. Dilutions of a plasmid extraction of a clone of the PCR product of a CTX-M-gr1 positive *E. coli* strain (E54) was used as calibration curve. DNA was diluted 1:100 times as PCR inhibition was still occasionally observed at a dilution of 1:10. Quantification of the CTX-M-gr1 was not possible as the Ct values of the positive samples fell out of the linear range of the calibration curve, and the assay was thus treated as a qualitative assay. As the amount of positive signals was low, samples that showed one or more positive PCR triplicates with a correct melt peak (87.5-88°C) in a 100x DNA dilution were confirmed in a second PCR on a 1:10 dilution of the DNA. If two or more of the three replicates of the second PCR were positive, and if melt curve analysis and gel electrophoresis confirmed the presence of correct amplicons, the sample was deemed positive. The great majority of the samples that showed one or more positive triplicate in the 1:100 diluted DNA (16 out of 19) was confirmed to be positive in the second PCR. None of the extraction blanks gave positive results, neither showed the non-template controls amplification.

Statistical analysis

Statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). Simple descriptive analyses were used to explore presence of CTX-M-gr1 in humans, dust and pigs over time. Farms were classified as CTX-M-gr1 positive in dust when CTX-M-gr1 was determined in at least one EDC sample. Farms were classified as CTX-M-gr1 positive in animals if CTX-M-gr1 was detected in an isolate from at least one pooled pig sample.

Generalized linear mixed models (PROC GLIMIX; SAS Institute, Inc.) adjusted for clustering at farm level and repeated measurements were used to calculate associations with CTX-M-gr1 carriage in humans. Our main determinant of interest was presence of CTX-M-gr1 in dust. Presence of CTX-M-gr1 in pigs and average number of hours working per week were also considered, since they were shown to have an effect by previous work based on cross-sectional data from this study[7]. All were analysed separately as well as together in a model. Potential confounders age, gender, and smoking were analysed univariately and selected for multivariate analysis when p-value was below 0.2. Model assumptions were checked with diagnostic plots.

Results

Human characteristics

During the first sampling moment, 142 people living and/or working on 34 of the 40 included pig farms participated. During the second sampling moment, 135 people living and/or working on 32 out of 39 participating pig farms were measured. In total, 131 people living and/or working on 32 pig farms participated in both sampling moments. Baseline characteristics of these 131 participants are presented in Table 1.

CTX-M-gr1 in humans

During the first sampling moment, six out of 142 participants carried CTX-M-gr1 versus four out of 135 participants during the second sampling moment. From the total of 131 participants who completed both sampling moments, only one person was a CTX-M-gr1 carrier during both sampling moments. In all human CTX-M-gr1 carriers, $bla_{\text{CTX-M-1}}$ was the determined gene type. Of the total of nine carriers (six farmers, three family members), eight participants reported to work at least 20 hours per week on the farm. During eight out of the ten positive human observations, pooled pig samples that originated from the corresponding farm were CTX-M-gr1 positive as well. All seven human carriers living on a farm with CTX-M-gr1 positive pigs reported to have daily contact with pigs. During six out of the ten positive human observations, dust samples originated from the corresponding farm were CTX-M-gr1 positive. None of the carriers

reported to have used antimicrobials, been hospitalized or have traveled to risk countries in the previous 12 months before both sampling moments.

Table 1. Baseline characteristics of participants who completed both sampling moments (n=131).

Human characteristics*	Frequency (%)
Gender	
Male	76 (58)
Female	55 (42)
Category	
Farmer	45 (34)
Family of farmer	70 (53)
Employee	16 (12)
Age	131 (mean 36, range 6-79)
Age <18 years	30 (23)
Age 18-65 years	98 (75)
Age >65 years	3 (2)
Average number of hours working on the farm per week	125 (mean 25, range 0-80)
0	39 (30)
1-20	25 (19)
≥20	61 (47)
Smoking	
Yes	11 (8)
No	120 (92)

*Measured at the start of the study period (first sampling moment). Differences in characteristics between the two sampling moments were minor.

CTX-M-gr1 in dust and pigs

The presence of CTX-M-gr1 in dust and pigs is listed in Table 2. During the first sampling moment, CTX-M-gr1 was detected in 11 out of 123 EDC's placed in the stables of 38 farms and in one of 34 EDC's placed in the house. During the second sampling moment, CTX-M-gr1 was detected in four out of 138 EDC's placed in the stables of 36 farms and in none of the 35 house EDC's. During the first and second sampling moment, ten out of 38 and three out of 36 farms were positive for CTX-

M-gr1 in dust. All three positive farms in the second sampling moment, were also positive in the first sampling moment. Quantification of CTX-M-gr1 was not possible, therefore we only presented binary results.

On 13 out of 40 and 11 out of 39 farms CTX-M-gr1 was present in pigs in the first and second sampling moment respectively. On eight out of ten farms (and 11 out of 13 farm observations) where CTX-M-gr1 positive farm EDC's were found, CTX-M-gr1 was detected in pigs simultaneously in time.

Association between ESBL carriage in humans and dust

Human CTX-M-gr1 carriage and presence of CTX-M-gr1 in dust were associated univariately (OR=12.4, 95% CI=2.7-57.1). Other determinants, univariately associated with human CTX-M-gr1 carriage, were presence of CTX-M-gr1 in pigs (OR=14.4, 95% CI=2.5-82.9) and average number of hours working on the pig farm per week (OR=1.04, 95% CI=1.01-1.07). When the three determinants were mutually adjusted in a multivariate analysis, the effect of working hours per week only changed marginally (OR=1.03, 95% CI=1.00-1.06). However, the effect sizes of presence of CTX-M-gr1 carrying pigs on the farm and presence of CTX-M-gr1 in dust were greatly reduced in the final model (OR=7.4, 95% CI=1.1-49.7 and OR=3.5, 95% CI=0.6-20.9 respectively). Gender was the only confounder considered for multivariate analysis (OR=4.6, 95% CI=0.8-25.9) but was not retained in the final model after adjustment for working hours. To evaluate dependency between the different effects in the final model, bivariate analyses were performed. Both presence of CTX-M-gr1 in dust and presence of CTX-M-gr1 in pigs declined in effect size when bi-variately analyzed (OR=5.3, 95% CI=1.1-26.2 and OR=7.2, 95% CI=1.2-41.5 respectively). In bivariate models together with the effect of working hours per week, there was a modest drop in the effect size of presence of CTX-M-gr1 in dust (OR=9.2, 95% CI=1.9-45.9). The change in effect size of presence of CTX-M-gr1 in pigs was minor (OR=12.8, 95% CI=2.2-74.4). The effect of average number of hours working on the pig farm per week hardly changed in any of the bivariate models. All associations are presented in Table 3.

Table 2. CTX-M-gr1 in humans, dust and pigs.

	First sampling moment			Second sampling moment		
Farm	**CTX-M-gr1 in humans**	**CTX-M-gr1 in dust**	**CTX-M-gr1 in pooled pig samples**	**CTX-M-gr1 in humans**	**CTX-M-gr1 in dust**	**CTX-M-gr1 in pooled pig samples**
1	0/5	0/4	0/10	0/6	0/4	0/10
2	0/1	1/2	9/10	1/1	2/4	3/10
3	0/1	0/4	1/10	0/1	0/4	0/10
4	0/9	0/2	1/10	0/9	0/4	1/10
5	2/4	2/4	5/10	0/4	1/4	3/10
6	NA	0/4	0/10	NA	0/4	0/10
7	0/2	0/4	1/10	0/2	0/4	1/10
8	0/4	0/4	0/10	0/4	0/4	0/10
9	NA	1/4	4/10	NA	0/4	0/10
10	0/2	0/3	0/10	0/2	0/4	0/10
11	0/1	0/2	0/10	0/1	0/4	0/10
12	0/3	NA	10/10	NA	NA	8/10
13	1/3	1/3	9/10	0/3	0/3	6/10
14	0/4	1/4	0/10	1/4	0/3	7/10
15	0/8	1/4	0/10	0/8	0/4	0/10
16	0/6	0/4	0/10	0/6	0/4	0/10
17	NA	1/4	9/10	NA	NA	0/9
18	0/4	0/3	0/10	0/4	0/3	0/10
19	0/9	0/2	0/10	0/8	0/4	0/10
20	0/3	0/4	0/10	0/2	0/4	0/10
21	0/3	0/3	0/10	0/3	0/3	0/10
22	0/2	1/2*	5/10	0/2	1/3	6/10
23	NA	0/2	0/10	NA	0/3	0/10
24	0/11	0/3[1]	0/10	1/11	0/4	0/10
25	0/1	NA	0/10	NA	NA	NA
26	0/5	0/4	1/10	0/5	0/4	1/10
27	0/2	0/1	0/10	0/2	0/4	0/10
28	0/2	0/2	0/10	0/1	0/6	0/10
29	NA	0/4	0/10	NA	0/4	0/10
30	0/7	0/2	0/10	0/8	0/3	0/10
31	2/5	1/3	8/10	1/5	0/4	5/10
32	NA	1/4	7/10	NA	0/4	6/10
33	0/5	0/3	0/10	0/3	0/3	0/10
34	0/7	0/4	0/10	0/7	NA	0/10
35	0/1	0/4	0/10	0/1	0/4	0/10
36	0/9	0/4	0/10	0/9	0/4	0/10
37	0/2	0/2	0/10	0/2	0/4	0/10
38	0/3	0/4	0/10	0/3	0/4	0/10
39	0/2	0/4	0/10	0/2	0/4	0/10
40	1/6	0/3	0/10	0/6	0/4	0/10

* CTX-M-gr1 was also determined in house EDC.
[1] Two living houses were sampled
NA = no samples were collected from these farms

Table 3. Longitudinal univariate and multivariate analyses for CTX-M-gr1 carriage in pig farmers, family members and employees.

Determinant	No* or mean	Univariate OR (CI)	Bivariate OR (CI)		Multivariate OR (CI)
Presence of CTX-M-gr1 in farm dust					
Yes	34	12.4 (2.7-57.1)	5.3 (1.1-26.2)	9.2 (1.9-45.9)	3.5 (0.6-20.9)
No	222	Ref.	Ref.	Ref.	Ref.
Presence of CTX-M-gr1 in pigs					
Yes	67	14.4 (2.5-82.9)	7.2 (1.2-41.5)	12.8 (2.2-74.4)	7.4 (1.1-49.7)
No	195	Ref.	Ref.	Ref.	Ref.
Average number of hours working on pig farm per week (per hour)	24±25	1.04 (1.01-1.07)	1.03 (1.00-1.06)	1.03 (1.00-1.06)	1.03 (1.00-1.06)
Per 10 hours		1.4 (1.1-1.9)	1.3 (1.0-1.8)	1.4 (1.0-1.8)	1.3 (1.0-1.8)
Age (per year)	37±17	1.02 (0.98-1.06)			
Gender					
Male	152	4.6 (0.8-25.9)			
Female	110	Ref.			
Smoking					
Yes	21	1.5 (0.2-14.9)			
No	239	Ref.			

*Based on total no of observations
Ref. = reference category

Discussion

In pig farmers, CTX-M-gr1 carriage was associated with exposure to CTX-M-gr1 containing dust and exposure to CTX-M-gr1 positive pigs. The observation that both variables were associated with human carriage, after mutual adjustment, leaves open the possibility that next to direct contact, airborne transmission plays a role as well. However, the number of human carriers was small and these findings need replication in a larger population sample. Moreover, presence of CTX-M-gr1 in dust and pigs were partially dependent, which is plausible because CTX-M-gr1 carrying pigs are shedding CTX-M-gr1 producing Enterobacteriaceae into the environment and CTX-M-gr1 might be picked up from the environment as well, which complicates the analysis.

Overall prevalence of human $bla_{CTX\text{-}M\text{-}1}$ carriage was 3.6%, which is more or less comparable to numbers found in two recent Dutch studies. In residents of Amsterdam and residents living in the vicinity of livestock farms 26 out of 1695 (1.5%) and 13 out of 2432 (0.5%) carried $bla_{CTX\text{-}M\text{-}1}$ respectively[17,18]. However, in pig farmers a $bla_{CTX\text{-}M\text{-}1}$ prevalence of 8% was seen. The number of farms where CTX-M-gr1 was detected in dust decreased from ten out of 38 farms to three out of 36 farms during the study period. The prevalence of CTX-M-gr1 in dust samples collected from the stables declined from 9% to 3%. This is in the same order of magnitude as a German study where 29 out of 282 dust samples harbored ESBL-producing *E. coli* (10.3%) of which most of the isolates (86 out of 106) belonged to CTX-M-gr1[9]. In a German study, cefotaxime resistant *E. coli* were present on ten out of 48 farms and 11% of all dust samples[10]. However, the number of farms with cefotaxime resistant *E. coli* in manure collected from the floor was much higher (40 out of 48 farms) than in the present study. In one out of the total of 69 EDC's placed in living houses, presence of CTX-M-gr1 was determined. Considering air exposure as a potentially relevant transmission route, this could partially explain the low carriership in people only living on the pig farm and not working in the stables (i.e. family members).

There was a considerable decrease in the number of farms where CTX-M-gr1 was detected in dust during the study period. At the same time, the number of farms where CTX-M-gr1 carrying pigs were present hardly changed over time. However, a reduction in pig prevalence (in terms of number of pooled pig samples) from 18% till 12% was seen. The proportion of positive EDC's on a farm was lower when the number of positive pooled pig samples was lower. Therefore, it seems likely that the amount of CTX-M-gr1 in dust must have been smaller during the second sampling round. It seems reasonable to assume that a lower sample prevalence is accompanied by a lower load of CTX-M-gr1 in the farm environment. As a consequence, more non-detects of CTX-M-gr1 in dust might have occurred during the second sampling

moment. Since the qPCR signals were close to the detection limit, CTX-M-gr1 levels in dust could have been too low to be detected. Possibly, this might have diluted the effect of air exposure, mostly due to the second sampling moment. The occurrence of non-detects is less likely for human and pooled pig samples since these were analyzed by culturing after using pre-enrichment. This approach has a very low detection limit, lower than qPCR applied for the EDCs. However, the low CTX-M-gr1 prevalence in humans together with the sample size is creating an issue of power. Overall, more conclusive evidence for the association between ESBL carriage in pig farmers and presence of CTX-M-gr1 in dust may have been conducted by increased power and quantitative results. In addition, fixed static spot measurements might be underestimating real exposure compared to mobile equipment. The EDC's were placed out of reach from pigs, while air levels of ESBL might be higher in the direct surroundings of pigs.

Exposure to CTX-M-gr1 was measured by analyzing dust and pig feces. No measurements were performed on hands, mouth, face or in the nose. Therefore, the significance of the exact transmission route is hard to determine. In a German short report no ESBL-producing Enterobacteriaceae were detected in the nares of pig farmers[19]. This result is rather difficult to interpret, since intestinal carriage in pig farmers nor presence of ESBL-producing Enterobacteriaceae in animals or the environment was assessed. Since dust particles are relatively large, CTX-M-gr1 containing dust might be ingested instead of inhaled, which further complicates the differentiation between transmission routes. In two Dutch studies, personal inhalable dust samples were obtained from pig farmers. Both studies showed an average exposure to dust of approximately 2.6 mg/m^3 [20,21]. Inhalable dust is the dust fraction that can penetrate the respiratory organ. Because most of the particulates are relatively large, they will be deposited mainly in the (upper) airways and ingested after deposition. Assuming an average working day of eight hours and respiratory minute volume of 6 L/m, it can be estimated that pig farmers inhale ~7.5 mg dust per day on average. Since ESBL genes have been detected in dust, transmission of ESBL through dust in air is not unlikely. Quantitative information about viable ESBL-producing Enterobacteriaceae content of dust is required to use this information for a quantitative risk analysis and explore the plausibility of this hypothesis relative to other transmission routes such as uptake through hand mouth contact.

Since qPCR detects DNA directly, there was no information available on the viability of the Enterobacteriaceae that produce the CTX-M-gr1 enzymes. However, ESBL-producing Enterobacteriaceae have been cultured from dust samples in other studies[9,10]. Furthermore, this study showed epidemiological associations between the presence of CTX-M-gr1 in dust, pigs and humans, but didn't take into account the molecular complexity of ESBL transmission fully, i.e. clonal transmission or horizontal

gene transfer through plasmids. However, previous work has shown that clonal transmission is relevant between pigs and humans on farms, although horizontal transfer can occur as well[7,8].

Results leave open the possibility of transmission through air as a relevant transmission route potentially leading to human ESBL carriage. If these results are confirmed in additional studies, personal preventive measures for pig farmers might need to involve general hygiene measures (changing clothing, hand washing) as well as reducing airborne particulate exposure. In addition, air exposure to ESBL might be involved in human to human transmission in other (clinical) settings as well. For improved exposure assessment and to gain more insight in potential transmission routes, quantified personal exposure measurements should be implemented in future research.

Conclusions

Results from this study suggest the possibility of airborne transmission of CTX-M-gr1 from pigs to humans.

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UND en VARKENSSLAGERU

Chapter 4

Risk factors for ESBL-producing *Escherichia coli* on pig farms: a longitudinal study in the context of reduced use of antimicrobials

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Abstract

The presence of extended-spectrum beta-lactamase-producing *Escherichia coli* (ESBL-*E. coli*) in food animals is a public health concern. This study aimed to determine prevalence of ESBL-*E. coli* on pig farms and to assess the effect of reducing veterinary antimicrobial use (AMU) and farm management practices on ESBL-*E. coli* occurrence on pig farms. During 2011-2013, 36 Dutch conventional pig farms participated in a longitudinal study (four sampling times in 18 months). Rectal swabs were taken from 60 pigs per farm and pooled per six pigs within the same age category. Presence of ESBL-*E. coli* was determined by selective plating and ESBL genes were characterized by microarray, PCR and gene sequencing. An extensive questionnaire on farm characteristics and AMU as Defined Daily Dosages per Animal Year (DDDA/Y) was available for the 6-month periods before each sampling moment. Associations between the presence of ESBL-*E. coli*-positive pigs and farm management practices were modelled with logistic regression. The number of farms with ESBL-*E. coli* carrying pigs decreased from 16 to ten and the prevalence of ESBL-*E. coli*-positive pooled pig samples halved from 27% to 13%. Overall, the most detected ESBL genes were $bla_{\text{CTX-M-1}}$, $bla_{\text{TEM-52}}$ and $bla_{\text{CTX-M-14}}$. The presence of ESBL-*E. coli* carrying pigs was not related to total AMU, but it was strongly determined by the presence or absence of cephalosporin use at the farm (OR=46.4, p=0.006). Other farm management factors, related with improved biosecurity, were also plausibly related to lower probabilities for ESBL-*E. coli*-positive farms (e.g. presence of a hygiene lock, pest control delivered by a professional). In conclusion, ESBL-*E. coli* prevalence decreased in pigs during 2011 and 2013 in the Netherlands. On pig farms, the use of cephalosporins was associated with the presence of ESBL-*E. coli* carrying pigs.

Introduction

A variety of ESBLs have been identified in Enterobacteriaceae derived from food-producing animals worldwide[1]. High antimicrobial use (AMU) and inappropriate use of cephalosporins in livestock production are considered to be associated with the emergence and high prevalence of ESBL-producing *Escherichia coli* (ESBL-*E. coli*) in animals[2]. Transmission of ESBL genes from animals to humans can occur through food or direct contact[3,4]. Infections with ESBL-*E. coli* are a major global public health concern[5].

Several European studies reported high proportions of pig farms where ESBLs were present. In Spain, ESBL-*E. coli* were detected in faecal samples collected from stable floors of eight out of ten farms[6]. Two German studies found ESBL-*E. coli* in faecal samples collected from pigs on 15 out of 17 and 26 out of 35 farms respectively[7,8]. In a Danish study ESBLs were detected in pigs on 15 out of 19 pig farms with high consumption of cephalosporins versus four out of 20 pig farms with no cephalosporin use[9].

A reduction in AMU, more specifically cephalosporins, is suggested to decrease ESBL-*E. coli* on pig farms[2,9,10]. Because of demands regarding reduction in AMU in livestock production by the Dutch government, the total consumption of antimicrobials by animals dropped drastically in the Netherlands since 2011[11-14]. Moreover, in 2011 the Dutch pig farm sector introduced a private initiative to stop the use of all cephalosporins (Dutch pig farms only used 3rd generation cephalosporins). Additionally, from January 2013, veterinarians were legally required to limit the use of 3rd/4th generation cephalosporins and fluoroquinolones to infections confirmed by bacteriological culture and susceptibility tests. As a consequence, at the vast majority of pig farms cephalosporins are not used anymore since 2011[12-15]. Although not studied until now, other management practices besides reduction in AMU might have an effect on the presence of ESBL-*E. coli* on pig farms as well.

The objectives of this longitudinal study were to determine the prevalence of ESBL-*E. coli* on pig farms and to assess the effect of AMU reduction and farm management practices on the presence of ESBL-*E. coli* on pig farms.

Materials and Methods

Study design

The design of the study has been described elsewhere[4,16]. Briefly, 36 multiplier pig farms (sows and piglets present), with or without finishing pigs, completed the study. Production types were classified in *farrowing* and *farrow-to-finish farms*. Farrowing

farms did not produce fatteners and they delivered piglets to finishing farms (with the exception of one farm delivering gilts for farrowing). Farrow-to-finishing farms integrated farrowing and finishing pig production and delivered fattening pigs to the abattoir. Additionally, a farm was defined as *open* when receiving external supply of gilts for at least once a year from at least one supplier, and as *closed* when there was no external supply of gilts.

Farms and veterinarians were visited at the start of the study by the researcher between March 2011 and September 2011. At four sampling moments over a period of 18 months (6-month intervals), rectal samples from 60 pigs were collected by the farm veterinarian, using sterile cotton-wool swabs (Cultiplast®) and sent refrigerated to the laboratory by courier. All animal age groups present were sampled: sows, gilts, suckling piglets, weaning piglets and finishing pigs. Rectal swabs were combined in ten pools (two per age category) of six pigs. When no finishing pigs were present, weaning piglets were sampled instead. Each pool consisted of an age group in the same pen. Approval from an animal ethics committee was not required. The collection of rectal swabs from animals was in compliance with the Dutch law for animal welfare and did not fall under the Dutch Experiments on Animals Act (1996) or Directive 2010/63/EU. At the first sampling moment (baseline measurements), a questionnaire was completed during a walk through survey by the farm veterinarian to identify which management aspects could be improved to reduce antimicrobial resistant bacteria. The questionnaire (S1 Table) contained items on farm characteristics, biosecurity, animal management and hygiene practices and can also be found elsewhere[16]. A tailor-made intervention protocol was developed by the veterinarian and the farmer. Interventions were focused on improving personnel and farm hygiene, changing animal contact structures, and reducing AMU (in a background of decreasing AMU nationwide due to government demands). At each sampling moment the farm questionnaire was filled out again to monitor changes in farm practices.

Laboratory analysis

All samples were analysed as described previously, namely pooled swabs were analysed for the presence of ESBL-producing Enterobacteriaceae by selective plating[4]. Samples were suspended in 10 ml peptone water and incubated overnight at 37°C. For screening of ESBL-producing Enterobacteriaceae, suspensions were cultured on selective agar plates (*Brilliance*™ ESBL Agar, Oxoid®) and incubated overnight at 37°C aerobically. When no growth was seen, plates were incubated another night at 37°C. Morphologically different colonies suspected of ESBL production were cultured individually on blood agar plates (Oxoid®) and incubated overnight at 37°C. In case of morphological uncertainty an oxidase test was performed before culturing. Bacterial species identification of the isolates was performed by MALDI/TOF (Bruker®).

For phenotypical confirmation of the presence of ESBL-producing Enterobacteriaceae, a 0.5 McFarland suspension was inoculated on a Mueller Hinton agar and a combination disc test (ROSCO®) including cefotaxime, cefotaxime+clavulanate, ceftazidime, ceftazidime+clavulanate, cefepime, and cefepime+clavulanate (*Neo-Sensitabs*™) was used (according to the guidelines of the manufacturer (http://www.rosco.dk)). Isolates were stored at -80°C.

In earlier cross-sectional research on the first sampling moment, most Enterobacteriaceae other than *E. coli* with ESBL phenotype did not harbor ESBL genes[4]. Therefore, only phenotypically confirmed ESBL-*E.coli* were selected for further molecular analysis in the remaining sampling moments. In all ESBL-*E. coli* the presence and characteristics of the of ESBL genes was identified by PCR and sequence analysis. DNA was isolated using UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories, Inc.) or DNeasy 96 Blood & Tissue Kit (Qiagen). Real-Time PCR (SybrGreen, Life Technologies), conventional PCR (BioMix Red, Bioline) and a bla_{CTX-M} group 1 specific PCR[17] was used to detect presence of the ESBL gene groups $bla_{CTX-M-1}$, bla_{TEM}, bla_{SHV} and bla_{CMY-2}. Isolates with a negative PCR result were analysed using ESBL microarray (Check-MDR CT101, Checkpoints, Wageningen) to detect other ESBL gene groups. DNA from PCR or ESBL microarray positive isolates was sequenced with group-specific primers to determine the exact gene type. DNA sequences were interpreted with Basic Local Alignment Search Tool (National Center for Biotechnology Information).

Data on antimicrobial use

The Defined Daily Dosage Animal per Year (DDDA/Y) is a standard weighted measure which can be interpreted as the number of days of antibiotic use per year for an average animal or animal place. A more detailed description on the calculation of DDDA/Y is described in the Netherlands Veterinary Medicines Authority report and by Bos et al.[11,14]. Data on AMU for the farms in this study have been described elsewhere[16]. In short, all antimicrobial prescriptions made to each farm were retrieved from the sector quality system national databases. AMU was expressed DDDA/Y per farm for the four periods preceding each sampling moment. Data was also available on wether the treatment was given individually or as group treatment. Since the use of cephalosporin was incidental, a new variable was created classifying farms with or without any cephalosporin use during the study period.

Data analysis

Statistical analyses were performed using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). Farms were classified as ESBL-positive if an ESBL gene was detected in at least one *E. coli* isolate from a pooled pig sample. One farm in the last sampling moment was classified ESBL-positive based on one phenotypically confirmed ESBL-*E. coli*, since the

isolate was lost before molecular analysis. Changes in presence of ESBL-producing *E. coli* on a farm and AMU over time were explored using simple descriptive statistics. DDDA/Y was $\log_2$ transformed because of its right-skewed distribution. Univariate longitudinal analysis was performed with AMU and farm questionnaire variables which had less then 10% missing values and more than 10% of farms present in each category. A total of 134 variables in the farm questionnaire (S1 Table) were selected together with AMU and cephalosporin use. The associations between presence of ESBL-producing *E. coli* on the farm and AMU, cephalosporin use and other farm variables was calculated with generalized linear mixed models (PROC GLIMIX; SAS Institute, Inc.) with random intercept for farms, taking into account the dependency of the data in a repeated measurements design. The univariate analysis was done for all the farms and for open and close farms separately; only associations from all farms with $p \leq 0.2$ from the questionnaire were presented. Pairwise Spearman correlations in questionnaire variables from the univariate analysis with $p \leq 0.1$ together with AMU and sampling time were checked to construct a full model. The final model was the result of a backward elimination from the full model, except for sampling time and AMU in DDDA/Y which were forced in the model during all steps because of special interest a priori. The final model retained variables significant at $p \leq 0.05$, again except for sampling moment and AMU. Model assumptions were checked with diagnostic plots. Variables from the full model at farm level were used to make a model at pooled pig sample level (i.e. modelling probabilities for a pooled pig sample to test ESBL-positive); this way we adjusted for age group of the animals. The latter model accounted for clustering at the farm level.

Results

Presence of ESBL-E.coli on pig farms

A description of the 36 farms is presented in Table 1. The number of farms where ESBL-*E. coli* carrying pigs were present decreased significantly from 16 farms at the beginning of the study (month 0) to ten positive farms in the last sampling moment (month 18). Nineteen farms were negative for ESBL-*E. coli* during the whole study (eight farrow-to-finish closed, five farrowing open, four farrow-to-finish open and two farrowing closed). Eight farms were ESBL-*E. coli*-positive in all sampling moments (six farrow-to-finish open, one farrow-to-finish closed and one open farrowing farm). Seven farms became negative during the study (three farrowing open, one farrow-to-finish open, two farrow-to-finish closed and one farrowing close). One farrow-to-finish open farm became ESBL-*E. coli*-positive during the course of the study. The median number of ESBL-*E. coli*-positive pooled samples among the ten collected per farm and per sampling time was 0 (IQR=0-3, percentile 95^{th}=8).

A pronounced and statistically significant drop in prevalence of ESBL-*E. coli* was observed over the study period. The proportion of ESBL-*E. coli*-positive pooled pig samples in all farms halved from 27% at the first to 13% at the last sampling moment. Farrow-to-finish open farms showed a clear higher prevalence as compared to other farm types (Figure 1).

ESBL-*E. coli* carriage significantly differed between the sampled age groups. Overall ESBL-*E. coli* prevalence in pooled pig samples ranged from 11.7% in (rearing) gilts to 24.2% in sucking piglets (Table 2). The prevalence decreased parallel across all age groups (results not shown). Mostly $bla_{CTX\text{-}M\text{-}1}$ genes were detected in pig isolates. Other ESBL genes found were $bla_{TEM\text{-}52}$, $bla_{CTX\text{-}M\text{-}14}$, $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}2}$ and $bla_{CTX\text{-}M\text{-}32}$ (Table 3).

Table 1. Farm characteristics.

		Median no. (interquartile range)	
Type of farm	**No. of farms**	**Sows**	**Fatteners**
All farms	36	350 (270-550)	773 (0-1950)
Open farms[a]	22	337 (300-500)	500 (0-1300)
Farrowing[b]	9	533 (350-800)	-
Farrow-to finish	13	314 (242-380)	1100 (600-2010)
Closed farms[a]	14	407 (232-698)	1400 (450-2725)
Farrowing[b]	3	439 (239-905)	-
Farrow-to finish	11	367 (200-673)	1892 (1025-2950)

[a] Farms were defined as open when they received external supplies of gilts ≥1 time per year from at least one supplier and as closed when they received no external supply of gilts.
[b] No fattening pigs present.

Table 2. Prevalence of ESBL-*E. coli* in pooled pig samples within different age groups.

Age group	Pooled pig samples (n)	Pooled pig samples with presence of ESBL-*E. coli* (n and %)
Sows	283	60 (21.2)
(Rearing) gilts	281	33 (11.7)
Suckling piglets[a]	285	69 (24.2)
Weaned piglets	318	66 (17.2)
Finishing pigs	183	31 (16.9)

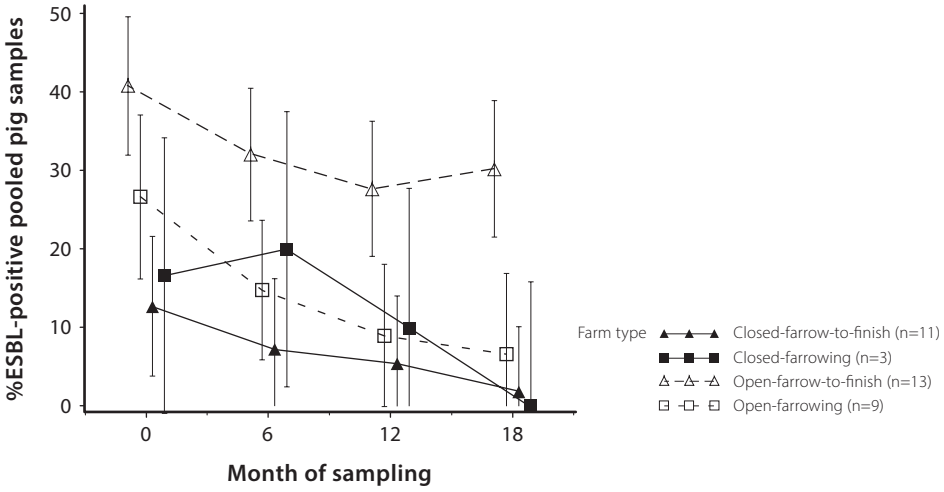
[a] Suckling piglets = pooled pig sample contained rectal swabs from one mother sow and five of her suckling piglets.

Table 3. Distribution of ESBL genes in isolates from pooled pig samples.

Sampling time	$bla_{CTX-M-1}$	bla_{TEM-52}	$bla_{CTX-M-14}$	$bla_{CTX-M-15}$	Other[a]	Total
0 mo	81[b]	24	18	11	5	139
6 mo	57	15	3	3	2	80[c]
12 mo	53	15	3			71
18 mo	42	20	3		1	66

[a] Other: CTX-M-2 (n=7), CTX-M-32 (n=1).
[b] $bla_{CTX-M-1}$ isolates were not tested for additional genes in the first sampling moment.
[c] One isolate was harbouring 2 ESBL genes.

Figure 1. Prevalence of ESBL-*E. coli*-positive pooled samples from pigs per farm type. Error bars indicate 95% CIs.



Evaluation of interventions: marked antimicrobial use reduction and minor changes in farm management

Farms considerably reduced AMU, likely as a result of the national benchmarking program for farms. A steady downward trend in $\log_2$ DDDA/Y, mirroring the overall national trend, was observed in all farm types except in farrowing open farms with a small (0.7%) increase in AMU (Figure 2). The AMU reduction was the highest in farrow-to-finish open farms (64%) and in closed farms (farrow-to-finish and farrowing) there was around a 40% reduction (Figure 2). Open farms used three times more antimicrobials as compared to closed farms (overall DDDA/Y of 9.7 and 3.1 respectively). The difference in overall AMU between open and closed farms was independent of the presence or absence of fattening

pigs as shown by a non-significant interaction term between external supply and type of production. Being a farrowing farm had a multiplicative effect with a two-fold increase in DDDA/Y in the strata of open and closed farms (overall DDDA/Y of 13.7, 7.7, 6.0 and 2.6 for open farrowing, open farrow-to-finish, closed farrowing and closed farrow-to-finish respectively).

During the whole study period, tetracyclines were the most used antimicrobial (37.6% of the total DDDA/Y), followed by penicillins (30.2%), trimethoprim/sulfonamides (12.3%), macrolides/lincosamides (12.0%) and polymyxins (4.6%). The last 3.3% corresponded mainly to combinations of antibiotics but also included cephalosporins, amphenicols, pleuromutilines and fluoroquinolones. Six farms used cephalosporins in the period preceding the first sampling moment, two of these farms also used these cephalosporins in the period between the first and second sampling moment. One farm only used cephalosporins in the period between the first and second sampling moment. DDDA/Y for cephalosporins varied from 0.06 to 0.39.

Almost all antimicrobial classes had a parallel decrease during the study having similar DDDA/Y percentages across all the periods preceding each sampling moment (Figure 3). Only macrolides had a slight increase in percentage of DDDA/Y during the study accompanied by a slight decrease in tetracyclines and trimethoprim/sulfonamides (Figure 3). Overall, 86% of the DDDA/Y were administered as (partial) herd treatment and 13.4% as individual treatment and these percentages did not significantly differ by period of study or type of farm (not shown).

Figure 2. Antimicrobial use by type of farm during the four periods (≈six months) before each sampling moment.GM and 95% CI from log2 DDDA/Y. AMU, antimicrobial use. Error bars indicate 95% CIs.

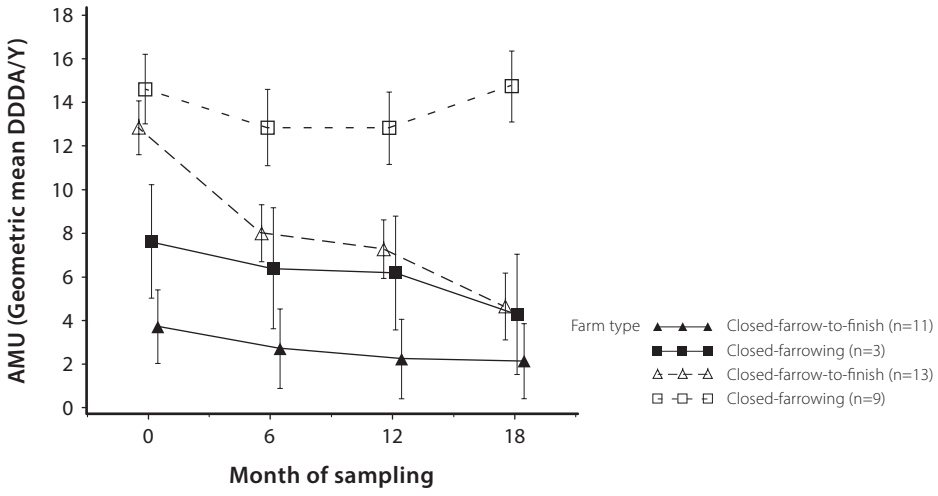
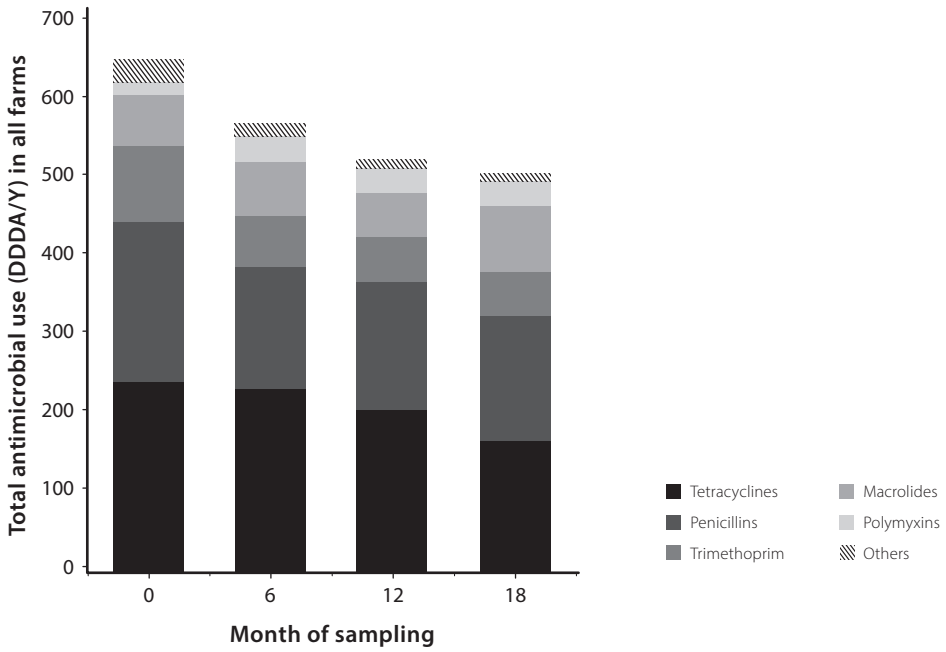


Figure 3. Proportions of antimicrobials used over the total DDDA/Y per farm type during the four periods (≈six months) before each sampling moment. DDDA/Y, defined daily dosages animal per year.



Farm management changes over time were modest; just 10% of the potential risk factors (median 9.7%, interquartile range (IQR)=6.0-12.3) changed during the study per farm. Thus, 27 farms had changes in less than 12 variables out of the total of 134. The median number of farms within a single change was 3 (IQR=1-4). Thus 75% of the changes occurred in four or less farms. No differences in changes over time were observed by the different farm types. Because of these limited and heterogeneous changes, an intervention effect of these changes could not be evaluated and we performed only a risk factor analysis.

Antimicrobial use and farm management practices related to presence of ESBL-*E. coli* in pig farms

Univariate ORs for the presence of ESBL-*E. coli* carrying pigs on a farm are presented in Table 4. The probability for a farm to have ESBL-*E. coli* carrying pigs was 24% higher per twofold increase in DDDA/Y, but this association was not statistically significant and did not change during the course of the study (i.e. there was a non-significant interaction between sampling moment and AMU for a farm to test ESBL-positive). Stratified analysis showed this positive relation in closed farms as well, but not in

open farms. Class specific DDDA/Y were not significantly associated to the presence of ESBL on pig farms. However, other variables regarding AMU were associated with ESBL-positivity of farms. When more than half of the treatments was provided to a group of pigs instead of an individual pig, the odds of a farm being ESBL positive was approximately four times higher. The use of cephalosporins at any time in the six months preceding and during the study period was significantly positively associated with the presence of ESBL-*E. coli* carrying pigs on a farm (OR=12.6, 95% CI=1.1-144.4) (Table 4).

Table 4. Univariate ORs for a pig farm to be ESBL-*E. coli*-positive.

		All farms		Open farms		Closed farms	
Determinant	**Category**	**N[b]**	**OR (95% CI)**	**N[b]**	**OR (95% CI)**	**N[b]**	**OR (95% CI)**
Farm characteristics							
No. sows	per 100 increase	144	0.69 (0.45-1.06)**	88	0.56 (0.30-1.05)**	56	0.84 (0.42-1.65)†
External supply of gilts	Open	88	6.0 (0.7-48.8)**	0	nc	56	nc
	Closed	56	Ref	88		0	
Type of production[a]	Farrow-to-finish	96	3.1 (0.4-25.4)†	52	10.3 (0.8-135.4)**	44	0.58 (0.00-72.26)†
	Farrowing	48	Ref	36	Ref	12	Ref
Water supply for animals	Public, from tap	46	0.12 (0.02-0.87)***	22	0.17 (0.01-2.61)*	24	0.15 (0.00-5.64)†
	Private source	94	Ref	63	Ref	31	Ref
Presence of goats in the farm	Yes	17	15.1 (0.8-271.8)**	10	27.2 (0.4-1863.5)*	7	28.7 (0.1-7904.0)†
	No	127	Ref	78	Ref	49	Ref
MRSA pool prevalence	per 10% increase	144	1.22 (0.94-1.58)*	88	1.18 (0.82-1.70)†	56	1.29 (0.76-2.19)†
Biosecurity							
Hygiene lock is the only entrance	Yes	81	0.21 (0.04-1.01)**	51	0.17 (0.02-1.22)**	30	0.17 (0.01-5.59)†
	No	62	Ref	37	Ref	25	Ref
Drivers do not enter the clean road	Yes	96	0.23 (0.05-1.18)**	50	0.21 (0.03-1.62)*	46	0.55 (0.02-18.37)†
	No	45	Ref	37	Ref	8	Ref
Dogs can enter the shed	Yes	29	5.0 (0.9-28.7)**	27	4.7 (0.7-34.0)*	2	nc
	No	115	Ref	61	Ref	54	
Removal of manure in summer	Manure stays <6 mo	123	0.21 (0.03-1.46)*	72	0.15 (0.02-1.44)**	51	nc
	Manure stays >6 mo	18	Ref	14	Ref	4	
Pest control is handed over to a professional organization	Yes	99	0.12 (0.02-0.75)***	60	0.26 (0.03-2.41)†	39	0.00 (0.00-0.26)***
	No	44	Ref	28	Ref	16	Ref

		All farms		Open farms		Closed farms	
Determinant	**Category**	**N[b]**	**OR (95% CI)**	**N[b]**	**OR (95% CI)**	**N[b]**	**OR (95% CI)**
Animal management and contact structure							
Foster sows can have pigs from more than one litter	Yes	75	2.5 (0.6-9.5)*	45	3.7 (0.7-19.5)*	30	0.97 (0.04-24.26)†
	No	57	Ref	34	Ref	23	Ref
Housing of gestating sows	Cubicle	69	3.3 (0.6-19.1)*	43	8.2 (1.0-68.7)***	26	0.35 (0.01-19.09)†
	Groups	69	Ref	41	Ref	28	Ref
Sick and cripple animals are taken care of in their own section[c]	Yes	29	4.7 (1.0-23.5)**	18	7.8 (1.0-59.5)***	11	0.68 (0.01-33.78)†
	No	103	Ref	59	Ref	44	Ref
Gloves always used when treating piglets	Yes	39	3.0 (0.6-15.9)*	19	4.0 (0.4-41.2)†	20	5.4 (0.2-141.9)†
	No	104	Ref	69	Ref	35	Ref
Tooth clipping in piglets	Yes	52	5.1 (0.9-29.0)**	35	5.0 (0.5-54.2)*	17	8.3 (0.2-337.6)†
	No	89	Ref	51	Ref	38	Ref
Antimicrobial use							
Antimicrobial use (log2DDDA/Y)[a] in 6 months preceding a sampling moment	per twofold increase	144	1.24 (0.84-1.84)†	88	0.88 (0.53-1.47)†	56	1.85 (0.73-4.66)*
Use of cephalosporins at any sampling moment	Yes	28	12.6 (1.1-144.4)***	24	3.92 (0.2-72.5)†	4	nc
	No	116	Ref	64	Ref	52	
Proportion of group treatments[d]	Above 0.5	100	4.0 (0.8-19.2)**	72	1.74 (0.22-13.63)†	28	7.5 (0.3-221.1)†
	Below 0.5	44	Ref	16	Ref	28	Ref

OR, odds ratio; Ref, reference category; nc, non-computable.

[a] Items evaluated irrespective of significance.

[b] Number of observations at all sampling times together (36 farms in four sampling times). Some variables have missing observations.

[c] Variable is not selected for multivariable analysis because of having >5% of missing values over the total number of possible observations n=144).

[d] Variable is not selected for multivariable analysis because of high correlation with antimicrobial use (spearman rho=0.7)

†P>0.2, * p≤0.2, ** p≤0.1, *** p≤0.05.

The presence of ESBL-*E. coli* carrying pigs was significantly less likely when water for the pigs was supplied from a public source instead of a private source (OR=0.1, 95% CI=0.0-0.9), when a hygiene lock was the only entrance on the farm (OR=0.2, 95% CI=0.0-1.0) and when pest control was carried out by a professional (OR=0.1, 95% CI=0.0-0.8). There was a trend (p-value between 0.05 and 0.1) for the presence

of ESBL-*E. coli* carrying pigs for the following determinants: external supply of gilts, presence of goats in the farm, drivers do not enter the clean road, dogs can enter the shed, sick and cripple animals are taken care of in their own section and tooth clipping in piglets (Table 4).

The results from the final model at farm and pool level are presented in Table 5. Presence of goats in the farm and the use of cephalosporins before and during the study period were risk factors for the presence of ESBL-*E. coli* carrying pigs on the farm (OR=49.2, 95% CI=1.7->999.9 and OR=46.4 95% CI=3.1-393.1 respectively). A hygiene lock as the only entrance to the pig farm was a protective factor (OR=0.1 95% CI=0.0-0.5).

The same factors were significantly associated to the presence of ESBL-*E. coli* in the model at the pooled pig sample level. Thereby, a significant decrease of ESBL-*E. coli* positive pooled pig samples from the first to the last sampling moment was found. The presence of ESBL-*E. coli* was significantly different between the separate age groups in the final model at the pooled pig sample level.

Table 5. Multivariate ORs for a pig farm to be ESBL-*E. coli*-positive (Model A) and for a pooled pig sample to be ESBL-*E. coli*-positive (Model B).

		Model A (farm level)			Model B (pooled pig sample level)		
Variable		**N**	**OR (95%CI)**	**P-value**	**N**	**OR (95%CI)**	**P-value**
Age group	gilts	NA	NA	NA	279	0.27 (0.14-0.52)	<0.001
	finishers				183	0.48 (0.24-0.94)	
	suckling piglets				283	1.64 (0.89-3.02)	
	weaned piglets				380	0.59 (0.33-1.04)	
	sows				281	Ref	
Sampling time	0 mo	36	3.01 (0.50-18.0)	0.498	352	5.4 (2.8-10.2)	<0.001
	6 mo	36	1.11 (0.19-6.49)		356	1.78 (1.00-3.18)	
	12 mo	36	1.12 (0.19-6.50)		358	1.07 (0.60-1.90)	
	18 mo	35	Ref		340	Ref	
Presence of goats in the farm	yes	17	49.2 (1.7->999.9)	0.024	169	4.02 (1.10-15.30)	0.042
	no	126	Ref		1237	Ref	
Antimicrobial use (log2DDDA/Y)	per twofold increase	134	1.35 (0.86-2.13)	0.192	1406	0.99 (0.76-1.30)	0.943
Use of cephalosporins at any sampling moment	yes	28	46.4 (3.1-393.1)	0.006	271	72.0 (5.8-903.1)	0.001
	no	115	Ref		1135	Ref	
Hygiene lock is the only entrance	yes	81	0.06 (0.01-0.47)	0.007	797	0.06 (0.02-0.27)	<0.001
	no	62	Ref		609	Ref	

All variables in the full model were weakly correlated (spearman rho<0.4). OR, odds ratio; Ref, reference category; NA, not applicable.

Discussion

This study suggests that the restriction in the use of cephalosporins has likely resulted in a decrease of ESBL-*E. coli* carriage on pig farms. ESBL-*E. coli* carriage in pigs significantly decreased during the study period. The observed steady reduction in total AMU did not explain these changes but the incidental use of cephalosporins was shown to be the most influential factor for ESBL-*E. coli* carriage of animals on farms. Additional farm management practices focused on improved biosecurity were also shown to play a role on the presence of ESBL-*E. coli* on pig farms.

In terms of ESBL-*E. coli* prevalence and gene types, other European studies have reported higher numbers of positive farms while $bla_{\text{CTX-M-1}}$ gene is the most commonly found type in livestock in Europe[1,6-9].

Despite a parallel decrease of total AMU and ESBL-*E. coli* prevalence during the study, AMU was not significantly associated with an increased likelihood of ESBL-*E. coli*-positive farms. Remarkably, when cephalosporins had been applied before or during the study, the probability for a farm to have ESBL-*E. coli*-positive pigs was dramatically increased. Although we have to acknowledge that the confidence interval of this association was wide, its significance directly calls for the well-known causal evidence attributed to the use of these drugs for the emergence of ESBLs[2,18]. Most of the farms in this study did not use cephalosporins, which is comparable to the use in the Dutch pig farm population in the same period (2011-2013)[11,14]. We can conclude that for curbing ESBL numbers, reducing or restricting the use of cephalosporins was more decisive than an overall AMU reduction. Thereby, it can be hypothesized that the overall decrease of ESBL-*E. coli* carriage in pigs in this study was also a delayed result of the possible reduction in the use cephalosporins before 2011. This is in line with the fact that the farms that did use cephalosporins in this study only used it in the first two sampling moments. Cephalosporins are relatively new drugs and unlike other historically long used drugs such as tetracyclines or penicillins, resistance to cephalosporins seems not to be permanently established in bacterial communities[19]. This means that ESBL resistance might be more rapidly reverted in comparison with other resistances, as suggested elsewhere[20].

To our knowledge, evidence for risk factors for presence of ESBL-*E. coli* on pig farms other than AMU is very limited. A recent cross-sectional study in Germany showed that some farm management and hygienic factors could be tackled to control cefotaxime resistant *E. coli*[21]. In our study, the set of selected determinants in the univariate analysis showed that apart of the restricted use of cephalosporins, additional measures focused on improving biosecurity and animal management measures could be an aid to control ESBL-*E. coli* occurrence in pig farms. The introduction of new animals on pig farms has been reported as a risk factor for antimicrobial resistance[16,22]. In

this study, a trend was seen for higher probability of ESBL-*E. coli* in farms with an external supply of pigs. In terms of animal age groups, the presence of ESBL-*E. coli* decreases over the production cycle; from suckling piglets to weaned piglets and finishing pigs, as it has been already reported by a Danish study[23]. The presence of goats in the farm as a risk factor was retained in the final model; the plausibility of this causal relationship is very doubtful and this could be just an incidental finding resulting from these farms being less strict in management and biosecurity practices (i.e. a proxy for a more poorly managed pig farm). A more specific protective factor in the multivariate model for ESBL-*E. coli*-positive farms was the hygiene lock as only entrance to the farm; it is quite plausible that this biosecurity measure might prevent the entrance of ESBL in the farm as suggested for other drug resistances [24]. Changes in management practices not regarding AMU were minor, therefore risk factors were probably detected more because of contrast between farms than contrast within farms over time.

We consider that results observed for our sample of farms can be generalized to the Dutch sector at large. Farms in the study contained different production types, and more importantly, their AMU was very close to national data in terms of total volumes, proportions of different antimicrobial families and proportions of individual and group animal treatments[14]. However, the differences between open and closed farms need to be cautiously interpreted since we lacked statistical power for a stratified analysis. The statistical power was also seriously compromised when a quantitative association with cephalosporins was assessed. Also, cephalosporins are only used in day-old piglets in the Netherlands. The DDDA/Y might be an underestimation because of the small total amount used due to the low weight of the piglets. Because of the limited use (in frequency and quantity) of these drugs during the study, we just evaluated their associations with ESBL-*E. coli* categorically. Moreover, we hypothesize that the use of cephalosporins at any point is a proxy for the cephalosporin use before 2011. Therefore excluding time variation in the use cephalosporins was justified.

Human ESBL carriage and direct contact with ESBL-*E. coli* carrying pigs is associated as shown by previous work[4]. This may pose a health risk for farmers and potentially for other humans with regular contact with this working population. Thereby ESBL-*E. coli* may be transmitted into the general population through the food chain[25]. The decreased ESBL-*E. coli* prevalence and the effect of cephalosporins, next to improved biosecurity and other farm management practices, showed that reduction of ESBL-*E. coli* on pig farms is possible. This might lead to reduced transmission of ESBL-*E. coli* from pigs to humans, which could be beneficial to public health.

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Supporting information

S1 Table. Farm questionnaire used in each of the four sampling moments in the longitudinal risk factor analysis for ESBL-*E. coli* carriage in pigs.

Question		Possible answers
General farm characteristics		
1.	Farm size: mean number of sows present per year *	No.
2.	Type of production*	Farrowing / Farrow-to-finish
3.	Mean number of fattener pigs present per year	No.
4.	Frequency of pig supply per year	No.
5.	Number of farms from which pigs are supplied per year	No.
6.	External supply of gilts more than once a year from at least one supplier (aggregated from questions 3 and 4) *	Open / Closed
7.	Complete all in-all out system is applied for closed farm*	Yes / No
8.	Frequency of removal of piglets	No.
9.	Frequency of removal of fattener pigs	No.
10.	Frequency of removal of rearing gilts	No.
11.	Frequency of removal of sows	No.
12.	Percentage of loss of weaned piglets per year	No.
13.	Average lactation period (days)	No.
14.	Mean number of weaned piglets per sow per year	No.
15.	Mean number of weaned piglets per litter	No.
16.	Percentage of loss of fattener pigs per year	No.
17.	Percentage of loss of sows per year	No.
18.	Mean growth per piglet per day	No.
19.	Mean growth per fattener pig per year	No.
20.	After delivering the leftover piglets are placed together	Yes / No
21.	After delivering there is a leftover piglets department	Yes / No
Biosecurity and hygiene status		
22.	Hygiene status of the farm	A/ B/ C/ D/ E/ F
23.	The farm owns an Specific pathogen free (SPF) status*	Yes / No
24.	SPF status for	App/ Aujeszky/ M.Hyo/ PRRSv/ None
25.	Housing of the gestating sows *	Cubicle / Groups
26.	Group size of the gestating sows*	Yes / No
27.	There are other animals present on the farm*	Yes / No
28.	There are also sheep present on the farm*	Yes / No
29.	There are also goats present on the farm*	Yes / No

30.	There are also cattle present on the farm*	Yes / No
31.	There are also horses present on the farm*	Yes / No
32.	There are also poultry present on the farm*	Yes / No
33.	No other farming animals are present on the farm	Yes / No
34.	Cats are able to enter the shed	Yes / No
35.	Dogs are able to enter the shed*	Yes / No
36.	Number of people working on the farm (including assisting family members)	Yes / No
37.	Biosecurity score (aggregated sum from questions 38, 42, 45,49, 90, 136 where yes=1 and no=0)*	0 to 6
38.	There is only one entrance to the farm, which is the hygiene lock, other doors are locked*	Yes / No
39.	A doorbell or phone number of the owner is clearly visible at the entrance of the farm. In this way it is possible to contact the people of the farm*	Yes / No
40.	The farm's terrain is paved and cleaned up *	Yes / No
41.	Silos are filled from the side of the dirty road*	Yes / No
42.	Pigs and personnel go outside during working activities*	Yes / No
43.	The hygiene lock consists of a clean and dirty part, separated by a passage shower*	Yes / No
44.	The lock does not contain a shower, but it does consist of a clearly separated clean and dirty part	Yes / No
45.	Showering is mandatory*	Yes / No
46.	If showering is not mandatory, everyone washes his or her hands before entering the farm	Yes / No
47.	Showering is not mandatory, however wearing farm-issued clothing is (pants and shirt	Yes / No
48.	Farmer and his co-workers use the hygiene lock in the same way visitors do*	Yes / No
49.	The farmer and his co-workers wash their hands before entering the farm*	Yes / No
50.	There is warm water available*	Yes / No
51.	There is soap available	Yes / No
52.	There is a clean towel present	Yes / No
53.	There are clean boots and overalls available*	Yes / No
54.	Overalls are washed daily*	Yes / No
55.	Overalls are washed	Daily/Weekly / Monthly/ Less than monthly
56.	On average, for how many months does the manure stay in the pits during the summer?*	<6 / >6
57.	On average, for how many months does the manure stay in the pits during the summer?*	<3 / 3-6 / 6-9 / >9
58.	On average, for how many months does the manure stay in the pits during the winter?*	<6 / >6
59.	On average, for how many months does the manure stay in the pits during the winter?*	<3 / 3-6 / 6-9 / >9
60.	Delivered animals are placed in quarantine for a certain period of time. This part has its own entrance and is not a part of the rest of the farm	Yes / No
61.	The quarantine has its own lock and clothing	Yes / No
62.	The quarantine is visited at the end of the day	Yes / No
63.	After delivery, the gilts arrive at an empty and cleaned section. This is not a quarantine	Yes / No
64.	When gilts are delivered, these animals do not arrive at an empty section or quarantine	Yes / No
65.	Piglets are delivered on the same day as fatteners	Yes / No
66.	Sperm is delivered on the dirty road, the cooling box is not brought on the farm terrain*	Yes / No

67.	There is a delivery room for materials and bagged goods. Materials are not delivered directly to the farm*	Yes / No
68.	Pest control is handed over to a professional organization*	Yes / No
69.	Birds are able to enter the sheds*	Yes / No
70.	Is there presence of rats and/or mice?*	Yes / No
71.	Animals have access to an outdoor run (e.g. after weaning)	Yes / No
72.	When pigs are moved, they have to go outside*	Yes / No
73.	There is a boarding platform for the sows, preventing the truck from parking directly against the shed*	Yes / No
74.	There is a boarding platform for the piglets, preventing the truck from parking directly against the shed*	Yes / No
75.	There is a boarding platform for the fatteners, preventing the truck from parking directly against the shed. (yes/no)*	Yes / No
76.	There is boarding platform for piglets and/or fatteners*	Yes / No
77.	The boarding location is not situated directly next to or beneath an air inlet*	Yes / No
78.	The border for the delivery of animals is a 100% clear and is also implemented this way*	Yes / No
79.	After delivery of the animals, the delivery platform is cleaned and disinfected immediately*	Yes / No
80.	The driver does not enter the clean road*	Yes / No
81.	Transport trucks are clean, empty and disinfected when they arrive on the farm to load the sows*	Yes / No
82.	The carcass storage is cooled and locked*	Yes / No
83.	The carcass cooler is situated on the dirty road*	Yes / No
84.	Small destruction materials can be thrown into the cooler from the clean road*	Yes / No
85.	There is a double number of barrels on the farm. So there is a surplus of barrels*	Yes / No
86.	After the destructor emptied the barrels, the barrels are cleaned and disinfected before retrieved*	Yes / No
87.	Rinsing water of cleaning barrels is discharged into the sewer	Yes / No
88.	When handling carcasses, gloves are always worn*	Yes / No
89.	When treating sick animals, gloves are always worn*	Yes / No
90.	When treating piglets, gloves are always worn*	Yes / No
91.	After someone entered a pen of the weaned piglets or the fatteners, hygienic measures are taken	Yes / No

Animal health management

92.	During gestation, vaccinations are implemented	Yes / No
93.	During lactation, vaccinations are implemented	Yes / No
94.	The piglets and/or fatteners are vaccinated*	Yes / No
95.	PRRSv vaccination is implemented	Yes / No
96.	Mycoplasma hyponeumoniae vaccination is implemented	Yes / No
97.	PCV2 vaccination is implemented	Yes / No
98.	APP vaccination is implemented	Yes / No
99.	Glässer vaccination is implemented	Yes / No
100.	The piglets are vaccinated without the use of a needle	Yes / No
101.	The teeth of the new-born piglets are clipped*	Yes / No

102.	The tails of the piglets are docked*	Yes / No
103.	The boar piglets are castrated	Yes / No
104.	All piglets are given an injection of antibiotics in their first week of life*	Yes / No
105.	When treating the piglets, gloves are worn	Yes / No
106.	The gloves are renewed:	After each litter/ After each section /Each day
107.	When treating the piglets, other hygiene measures are taken in order to prevent the transfer of infection from one to the other litter*	Yes / No
108.	Needles for vaccination of sows are renewed:*	Once a day /
109.	Needles for vaccination of piglets and/or fatteners are renewed per pen*	Once a week / When necessary
110.	Needles for vaccination of piglets and/or fatteners are renewed per compartment*	Yes / No
111.	At the end of the day, the syringes are cleaned:	Yes / No
112.	There is a sick-bay present*	Daily, rinsing with cold water / Taken apart and with water and soap / Dishwasher / Not cleaned
113.	The sick-bay is used as a sick-bay	Yes / No
114.	In the sick-bay, different ages are present	Yes / No
115.	Animals enter and exit a sick-bay (back to the farm)	Yes / No
116.	The sick-bay is visited at the end of the day	Yes / No
117.	There is a care option for sick and cripple animals at their own group/section*	Yes / No

Animal contact structure

118.	The sows are housed in stable groups*	Yes / No
119.	Piglets are placed per litter*W	Yes / No
120.	Some piglets are reared motherless*	Yes / No
121.	After the third day, piglets can still be switched*	Yes / No
122.	Foster sows are used*	Yes / No
123.	When creating foster sows, different litters of piglets are moved up to a different sow	Yes / No
124.	Separation between piglet cages is open*	Yes / No
125.	Separation between piglet cages is taken up by the feeder and/or trough, which is shared between the animals*	Yes / No
126.	Supervision of the animals from the central hall way	Yes / No
127.	Separation between cages fatteners is open*	Yes / No
128.	Separation between cages fatteners is taken up by the feeder and/or trough, which is shared between the animals*	Yes / No
129.	Separation between cages for sows is open*	Yes / No
130.	Separation between cages for sows is taken up by the feeder and/or trough, which is shared between the animals*	Yes / No
131.	Carcasses are placed on the ground in the section*	Yes / No
132.	Carcasses are placed on the ground in the central hall way*	Yes / No

133.	Cadaver bags are used*	Yes / No
134.	Considering hygienic measures, direction of work is from young to old *	Yes / No
135.	Sows, piglets and fatteners are different components within the farm. Each component makes use of different clothing and materials*	Yes / No

Cleaning and disinfection

136.	All farm sections are cleaned and disinfected *	Yes / No
137.	All farm sections are disinfected *	Yes / No
138.	All farm sections are cleaned with soaking agents*	Yes / No
139.	Farrow compartment hygiene (aggregated variable from questions 143 and 144)*	Disinfection and soaking / Just soaking / None
140.	Farrow passage hygiene (aggregated variable from questions 148 and 149)*	Disinfection and or soaking / None
141.	Farrowing section is cleaned with cold water	Yes / No
142.	Farrowing section is cleaned with warm water	Yes / No
143.	Farrowing section is cleaned with soaking agent	Yes / No
144.	Farrowing section is cleaned with disinfection agent	Yes / No
145.	After cleaning farrowing section, there is a dry period of at least 24 hours	Yes / No
146.	Farrowing section is cleaned by sweeping	Yes / No
147.	Farrowing passage is cleaned with soaking agent	Yes / No
148.	Farrowing passage is cleaned with disinfection agent	Yes / No
149.	Piglets compartment hygiene (aggregated variable from questions 153 and 154)*	Disinfection and soaking / Just soaking / Just disinfection / None
150.	Piglets passage hygiene (aggregated variable from questions 157 and 158)*	Disinfection and soaking / Just soaking / None
151.	Piglets section is cleaned with cold water	Yes / No
152.	Piglets section is cleaned with warm water	Yes / No
153.	Piglets section is cleaned with soaking agent	Yes / No
154.	Piglets section is cleaned with disinfection agent	Yes / No
155.	After cleaning piglets section, there is a dry period of at least 24 hours	Yes / No
156.	Piglets section is cleaned by sweeping	Yes / No
157.	Piglets passage is cleaned with soaking agent	Yes / No
158.	Piglets passage is cleaned with disinfection agent	Yes / No
159.	Fatteners compartment hygiene (aggregated variable from questions 163 and 164)	Disinfection and soaking / Just soaking / Just disinfection / None
160.	Fattener passage hygiene (aggregated variable from questions 167 and 168)	Disinfection and or soaking / None
161.	Fattener section is cleaned with cold water	Yes / No
162.	Fatteners section is cleaned with warm water	Yes / No
163.	Fatteners section is cleaned with soaking agent	Yes / No
164.	Fatteners section is cleaned with disinfection agent	Yes / No

165.	After cleaning fatteners section, there is a dry period of at least 24 hours	Yes / No
166.	Fatteners section is cleaned by sweeping	Yes / No
167.	Fatteners passage is cleaned with soaking agent	Yes / No
168.	Fatteners passage is cleaned with disinfection agent	Yes / No
169.	Gilts compartment hygiene (aggregated variable from questions 173 and 174)*	Disinfection and or soaking / Just soaking / None
170.	Gilts passage hygiene (aggregated variable from questions 177 and 178)*	Disinfection and or soaking / None
171.	Gilts section is cleaned with cold water	Yes / No
172.	Gilts section is cleaned with warm water	Yes / No
173.	Gilts section is cleaned with soaking agent	Yes / No
174.	Gilts section is cleaned with disinfection agent	Yes / No
175.	After cleaning gilts section, there is a dry period of at least 24 hours	Yes / No
176.	Gilts section is cleaned by sweeping	Yes / No
177.	Gilts passage is cleaned with soaking agent	Yes / No
178.	Gilts passage is cleaned with disinfection agent	Yes / No
179.	Mating compartment hygiene (aggregated variable from questions 183 and 184) *	Disinfection and or soaking / None
180.	Mating passage hygiene (aggregated variable from questions 187 and 188)*	Disinfection and or soaking / None
181.	Mating section is cleaned with cold water	Yes / No
182.	Mating section is cleaned with warm water	Yes / No
183.	Mating section is cleaned with soaking agent	Yes / No
184.	Mating section is cleaned with disinfection agent	Yes / No
185.	After cleaning mating section, there is a dry period of at least 24 hours	Yes / No
186.	Mating section is cleaned by sweeping	Yes / No
187.	Mating passage is cleaned with soaking agent	Yes / No
188.	Mating passage is cleaned with disinfection agent	Yes / No
189.	Gestation shed compartment hygiene (aggregated variable from questions 193 and 194)*	Disinfection and or soaking / None
190.	Gestation shed passage hygiene (aggregated variable from questions 197 and 198)*	Disinfection and or soaking / None
191.	Gestation shed is cleaned with cold water	Yes / No
192.	Gestation shed is cleaned with warm water	Yes / No
193.	Gestation shed is cleaned with soaking agent	Yes / No
194.	Gestation shed is cleaned with disinfection agent	Yes / No
195.	After cleaning gestation shed, there is a dry period of at least 24 hours	Yes / No
196.	Gestation shed is cleaned by sweeping	Yes / No
197.	Gestation passage is cleaned with soaking agent	Yes / No
198.	Gestation passage is cleaned with disinfection agent	Yes / No

Workflow, feed and water supply

199.	Work is visibly done with a week planner*	Yes / No
200.	Work is visibly done with a day planner*	Yes / No
201.	There are protocols present in the shed (work flows) *	Yes / No
202.	The date of placement is present on the section doors *	Yes / No
203.	Farm treatment plan recorded and stored*	Yes / No
204.	A medical prescription with dosage is present on the farm*	Yes / No
205.	Farrowing sows are fed with broth*	Yes / No
206.	Farrowing sows are fed with dry feed*	Yes / No
207.	Farrowing sows are fed with milk	Yes / No
208.	Farrowing sows are fed with mush/pulp	Yes / No
209.	Dry and gestating sows are fed with broth*	Yes / No
210.	Dry and gestating sows are fed with dry feed*	Yes / No
211.	Dry and gestating sows are fed with milk	Yes / No
212.	Dry and gestating sows are fed with mush/pulp	Yes / No
213.	Gilts are fed with broth*	Yes / No
214.	Gilts are fed with dry feed*	Yes / No
215.	Gilts are fed with milk	Yes / No
216.	Gilts are fed with mush/pulp	Yes / No
217.	Piglets with sow are fed with broth*	Yes / No
218.	Piglets with sow are fed with dry feed*	Yes / No
219.	Piglets with sow are fed with milk*	Yes / No
220.	Piglets with sow are fed with mush/pulp*	Yes / No
221.	Weaned piglets are fed with broth*	Yes / No
222.	Weaned piglets are fed with dry feed*	Yes / No
223.	Weaned piglets are fed with milk	Yes / No
224.	Weaned piglets are fed with mush/pulp*	Yes / No
225.	Fatteners are fed with broth*	Yes / No
226.	Fatteners are fed with dry feed	Yes / No
227.	Fatteners are fed with milk	Yes / No
228.	Fatteners are fed with mush/pulp	Yes / No
229.	Animals get water mainly from:*	Public source, tap/ Private source
230.	Water medication is possible via a dosator*	Yes / No
231.	Water medication is possible per section*	Yes / No
232.	A separate medication pipe is present on the farm*	Yes / No
233.	The water pipe is cleaned*	Yes / No
234.	In the farrowing section drinking water is just supplied via a nipple*	Yes / No
235.	In the farrowing section drinking water is mainly supplied via a nipple*	Yes / No

236.	In the farrowing section drinking water is mainly supplied via a water bowl*	Yes / No
237.	In the piglet section drinking water is just supplied via a nipple*	Yes / No
238.	In the piglet section drinking water is mainly supplied via a nipple*	Yes / No
239.	In the piglet section drinking water is just supplied via water bowl*	Yes / No
240.	In the piglet section drinking water is mainly supplied via a water bowl*	Yes / No
241.	In the fattener section drinking water is mainly supplied via a nipple*	Yes / No
242.	In the fattener section drinking water is just supplied via water bowl*	Yes / No
243.	In the fattener section drinking water is mainly supplied via a water bowl*	Yes / No
244.	In the (rearing) gilt section drinking water is mainly supplied via a nipple*	Yes / No
245.	In the (rearing) gilt section drinking water is mainly supplied via a water bowl*	Yes / No
246.	In the mating section drinking water is mainly supplied via a nipple*	Yes / No
247.	In the mating section drinking water is mainly supplied via a water bowl*	Yes / No
248.	In the gestation shed drinking water is mainly supplied via a water bowl*	Yes / No

* Variables with less than 10% missing values, at least 10% of farms in each category considered in the statistical analyses.


UND en VARKENSSLAGERU

Chapter 5

Dynamics of ESBL-producing *Escherichia coli* in pigs and pig farmers – A longitudinal study

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Abstract

Extended-spectrum beta-lactamase-producing *Escherichia coli* (ESBL-producing *E. coli*) represent a public health hazard due to limited therapeutic options for treatment of infections. Direct contact with pigs is considered a risk factor for human ESBL-producing *E. coli* carriage through occupational exposure. Objectives were to determine the dynamics in prevalence and genetic characteristics of ESBL-producing *E. coli* in humans and pigs longitudinally within the same sample of pig farms. In addition, we investigated transmission events between pig farmers and their pigs, based on genetic relatedness and longitudinal epidemiological associations. On 39 conventional pig farms, faecal samples from 60 pigs per farm and in total 146 farmers, family members and employees were collected during four sampling moments with a six month interval. The presence of ESBL-producing *E. coli* was determined by selective plating. ESBL genes were identified by PCR, microarray and gene sequencing. Genetic characteristics of plasmids and strains were determined by PCR-based replicon typing (PBRT), plasmid multilocus sequence typing (pMLST), and multilocus sequence typing (MLST). Personal and farm characteristics were collected using questionnaires. ESBL genes were present in pigs at least once on 18 out of 39 farms and in 17 out of 146 farmers, family members and employees. Of all 417 ESBL-producing *E. coli* isolates, $bla_{\text{CTX-M-1}}$, $bla_{\text{TEM-52}}$, and $bla_{\text{CTX-M-14}}$ were the most frequently observed genes in both pigs (n=261, n=74 and n=27 respectively) and humans (n=25, n=3, and n=3 respectively). A great variety in plasmid (sub)types and *E. coli* sequence type (ST) was seen. On seven farms, genetic similarity between human and pig derived isolates was seen in gene, plasmid (sub)type and ST, suggesting clonal transmission. On four farms, similarity was seen in gene and plasmid subtype, leaving open the possibility of horizontal gene transfer. Five pig farmers carried an ESBL repeatedly, of which two carried an identical combination of gene, plasmid subtype and ST over time. Human ESBL carriage was associated to both presence of ESBL in pigs (OR=8.7, 95% CI=3.5-25.6) and average number of hours working on the pig farm per week (OR=1.04, 95% CI=1.02-1.06). Although transmission of ESBL from pigs to pig farmers did occur, prolonged carriage was observed incidentally.

Introduction

Livestock, including pigs, can carry extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae[1]. ESBL-producing Enterobacteriaceae can be transmitted from pigs to humans living and/or working on farms through direct contact with pigs[2-5]. Although duration of ESBL carriage has been described in the general population, travellers and health care setting[6-8], longitudinal data from other high risk populations, such as farming communities, is sparse. The first objective of this study was to determine the presence and genetic characteristics of ESBL-producing *E. coli* in pigs and humans longitudinally, including persistence of ESBL-producing *E. coli* in pig farmers that repeatedly carried ESBL. The second objective was to determine the association between human ESBL carriage and the presence of ESBL in pigs. Genetic relatedness was investigated to determine similarity between ESBL-producing *E. coli* from human and pigs within the same farm and over time.

Materials and Methods

Study design

The design of the study has been described partially in previous studies[2,9]. Briefly, 39 Dutch conventional pig farms were enrolled between 2011 and 2013. During four repeated sampling moments with a six-month interval, fecal samples and questionnaires were obtained from 146 farmers, their family members, and employees on a voluntary base from 34 farms. To assess potential exposure to ESBL in pigs, rectal swabs from 60 pigs on all 39 farms were collected by veterinarians at each sampling moment as well. Rectal swabs were pooled into ten pools of six pigs each per farm. Human participants filled out questionnaires on general characteristics, farm activities and duration of animal contact. The Medical Ethical Committee of the University Medical Centre Utrecht approved the study protocol (no. 10-471/K). All participants gave written informed consent.

Laboratory analysis

Both human and pooled pig samples were analysed for the presence of ESBL-producing *E. coli* by plating on selective agar plates. ESBL genes were identified and characterized by means of PCR, microarray and gene sequencing for each distinctive colonial morphotype (one to four per sample). All ESBL-harbouring isolates from humans and a maximum of five (similar ESBL gene harbouring) isolates of their pigs were selected for further molecular analysis. Plasmids carrying the ESBL genes were determined using a transformation-based approach. PCR-based replicon typing

(PBRT), plasmid multilocus sequence typing (pMLST), and multilocus sequence typing (MLST) were performed to check for genetic similarities on the ESBL-encoding plasmid and harbouring strain level, respectively. All technical details are described elsewhere[2,9,10].

Statistical analysis

Statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). Descriptive analyses were used to explore presence of ESBL genes in *E. coli* isolates from humans and pigs over time. Farms were classified as ESBL positive when ESBL was determined in at least one obtained pig isolate. Generalized linear mixed models (PROC GLIMMIX; SAS Institute, Inc.) adjusted for clustering at farm level and repeated measurements were used to calculate associations between ESBL carriage in humans and potential determinants. Only observations from humans that participated during at least three sampling moments were included in the model. Determinants considered were the presence of ESBL in pigs and the average number of hours working per week, which were analysed separately as well as together in a model. Potential confounders age, gender, and smoking were analysed univariately and selected for multivariate analysis when p-value was below 0.2. Model assumptions were checked using diagnostic plots.

Results

Prevalence of ESBL-producing *E. coli* in humans and pigs

ESBL genes were detected in *E. coli* isolates from 17 participants (13 pig farmers and four family members) at one or more sampling moment(s) (Table 1). From the total of 542 observations in humans, 23 ESBL positive observations were seen. Human prevalence over all sampling moments was 4% (CI 95%) and ranged from 6% (95% CI 2-9%) at the beginning to 2% (95% CI 0-5%) at the last sampling moment (8/141, 7/137, 5/135 and 3/129 in time order). Overall, prevalence of farms where ESBL-producing *E. coli* was present in pigs was 34% and ranged from 44% to 28% between the first and last sampling moment (17/39, 13/39, 12/39, 11/39 in time order). Out of 39 farms, 18 (46%) were positive for ESBL-producing *E. coli* in pigs at one or more sampling moment(s), of which nine farms (23%) at all four sampling moments. On 21 farms (54%), pigs were negative for ESBL-producing *E. coli* isolates at all sampling moments.

Table 1. ESBL genes in human and pig isolates on pig farms (only positive observations are listed).

		ESBL genes			
Farm	**Origin**	**0 months[1]**	**6 months**	**12 months**	**18 months**
1*	Farmer		CTX-M-1 (2)	CTX-M-1 (1)	CTX-M-1 (3)
	Pigs	CTX-M-1 (16)[2]	CTX-M-1 (12)	CTX-M-1 (3)	CTX-M-1 (10)
2	Pigs	CTX-M-1 (1)			
3	Pigs	CTX-M-1 (1) CTX-M-2 (4) TEM-52 (6)	CTX-M-2 (2) TEM-52 (3)	CTX-M-1 (1) TEM-52 (4)	CTX-M-2 (1) TEM-52 (8)
4*	Farmer a	CTX-M-1 (1)			
	Farmer b	CTX-M-1 (1)	CTX-M-1 (1)		
	Pigs	CTX-M-1 (7)	CTX-M-1 (6)	CTX-M-1 (3)	
5*	Farmer		CTX-M-1 (2)		CTX-M-14 (1)
	Pigs	CTX-M-1 (1) CTX-M-14 (10)	CTX-M-1 (3) CTX-M-14 (2)	CTX-M-1 (1) CTX-M-14 (2)	CTX-M-1 (2) CTX-M-14 (3) TEM-52 (1)
6	Pigs	CTX-M-1 (4)			
7	Pigs	CTX-M-14 (1)			
8	Pigs	CTX-M-1 (16)	CTX-M-1 (13)	CTX-M-1 (13)	CTX-M-1 (8)
9*	Farmer	CTX-M-1 (1)	CTX-M-15 (1)		-[3]
	Pigs	CTX-M-1 (13)	CTX-M-1 (7) CTX-M-15 (1)	CTX-M-1 (9)	CTX-M-1 (3)
10*	Farmer			CTX-M-1 (2)	
	Pigs			CTX-M-1 (9)	CTX-M-1 (12)
11	Farmer		CTX-M-15 (1)		
12	Pigs	TEM-52 (1)	CTX-M-1 (1) TEM-52 (1)		TEM-52 (5)
13	Pigs	CTX-M-15 (11)	CTX-M-1 (1) CTX-M-15 (2)		
14	Family member		CTX-M-1 (2)		-
15	Farmer				CTX-M-2 (1)
16*	Farmer		CTX-M-1 (1)		
	Pigs	CTX-M-1 (6)	CTX-M-1 (9)	CTX-M-1 (8)	CTX-M-1 (6)
17	Family member			CTX-M-1 (1)	
18*	Farmer a			TEM-52 (1)	
	Farmer b	TEM-52 (2)			
	Pigs	CTX-M-1 (1) TEM-52 (14)	CTX-M-1 (1) TEM-52 (11)	CTX-M-1 (1) TEM-52 (10)	TEM-52 (4)

Farm	Origin	ESBL genes			
		0 months[1]	6 months	12 months	18 months
19*	Farmer	CTX-M-1 (1)		CTX-M-1 (2)	
	Family member	CTX-M-1 (1)			
	Pigs	CTX-M-1 (10) CTX-M-32 (1)	CTX-M-1 (6)	CTX-M-1 (5)	CTX-M-1 (2)
20	Pigs	CTX-M-1 (11) TEM-52 (2)	CTX-M-1 (6)	CTX-M-1 (7) TEM-52 (1)	CTX-M-1 (5) TEM-52 (2)
21	Pigs	TEM-52 (1)			
22*	Farmer	CTX-M-14 (2)			
	Pigs	CTX-M-14 (7)	CTX-M-1 (1) CTX-M-14 (1)	CTX-M-14 (1)	
23	Family member	CTX-M-1 (3)			

* Within these farms the presence of the same ESBL genes was established in both pig farmers and their pigs in one or more sampling moment(s).

[1] All human and pig *bla*CTX-M-1 isolates collected in T0 were not tested for the presence of additional genes.

[2] The number indicates the total number of distinctive colonial morphotypes tested that carried the ESBL gene.

[3] No sample(s) were obtained.

ESBL genes

In total, 417 ESBL-producing *E. coli* were recovered, of which 34 from the 17 human participants and 383 from pigs. Among them, four ESBL gene types were identified belonging to either bla_{CTX-M} or bla_{TEM} gene families. The $bla_{CTX-M-1}$ gene was predominant among the recovered isolates and was detected in 25 out of 34 human (74%) and 261 out of 383 pig isolates (68%). 12 out of 17 (71%) participants positive for the presence of ESBL-producing *E. coli* carried the $bla_{CTX-M-1}$ gene at least once. Other frequently found ESBL genes were bla_{TEM-52} [n=74 (19%) and n=3 (9%) isolates in pigs and humans, respectively], $bla_{CTX-M-14}$ [n=27 (7%) and n=3 (9%)] and $bla_{CTX-M-15}$ [n=14 (4%) and n=2 (6%)]. On several farms diverse ESBL gene types were found in pigs within the same sampling moment and/or the present gene types could differ over time within the same farm. In most cases, the ESBL gene type found in pig farmers was identical to the predominantly or exclusively detected ESBL gene type in pig isolates on the same farm. Detailed data regarding ESBL genes in humans and pigs per farm and sampling moment are listed in Table 1.

Plasmid (sub)types and *E. coli* sequence types

A total of 104 ESBL-harbouring isolates (34 human and 70 pig isolates) were selected for further genetic characterisation of plasmids and strains. This selection included isolates detected on nine farms where ESBL genes were present in both humans and pigs. From these farms, all human ESBL-producing *E. coli* isolates (n=26) and a

maximum of five randomly selected identical ESBL gene-harbouring *E. coli* isolates from pigs within the same farm were included (Table 2a). In addition, human derived ESBL-producing *E. coli* isolates detected on five farms without ESBL genes present in their pigs were included (n=8) (Table 2b).

On seven farms, at least one of the human and one of the pig isolates within the same farm and sampling moment were identical in gene, plasmid (sub)type and *E. coli* ST (farm 1, 4, 5, 9, 10, 18, 19). On four farms, human and pig derived ESBL-harbouring isolates were identical in gene and plasmid subtype within the same sampling moment (farm 1, 16, 19, 22).

On farm 1, 4, 5, 9, 10, 16, and 19 $bla_{CTX\text{-}M\text{-}1}$ (n=70) was identified in humans and pigs, all encoded on IncI1 plasmid subtypes (pST7/pCC7 [n=43 (61%)], pST3/pCC3 [n=20 (28%)], pST38/pCC3 [n=6 (9%)] and $pST278_{SLV}$ [n=1 (2%)]). On farm 10, 16 and 19 plasmid subtype pST7/pCC7 was exclusively identified in human and pig derived isolates. On farm 5 and 22, $bla_{CTX\text{-}M\text{-}14}$ (n=11) was detected in humans and pigs located on plasmids assigned to F2:A-:B- (n=8) F2:A-:B13 (n=1), F2:A-:B42 (n=1) and ColE2 (n=1) (sub)types. On farm 18, the $bla_{TEM\text{-}52}$ gene was detected in isolates from both humans and pigs, in all of which the gene was present on IncX1 plasmids (n=13). On farm 9, $bla_{CTX\text{-}M\text{-}15}$ (n=2) was detected in one human and one pig derived isolate, located on a F-:A20:B1 plasmid subtype and the chromosome respectively.

E. coli isolates were distributed into 38 different sequence types (STs), each comprised of one to 12 isolates, with ST453/CC86 (n=12), ST10/CC10 (n=10) and ST58/CC155 (n=9) being the predominant ones, while isolates belonging to human-related epidemic clones (i.e. ST101, ST410, ST711, ST744) were also identified. The number of identified STs per farm varied between one (farm 9) to eight (farm 1), with the presence of diverse *E. coli* STs circulating within the majority of the farms. On farm 4, 12 out of 13 isolates harbouring $bla_{CTX\text{-}M\text{-}1}$ on different IncI1 plasmid types obtained during two sampling moments belonged to ST453.

On five farms, human participants carried an ESBL without ESBL being present in pigs on the farm (Table 2b). In these isolates, the ESBL genes were located on diverse IncI1 plasmid subtypes (pST3/pCC3, pST58/pCC58, pST12/pCC12, Untypable pST), an F plasmid subtype (F2:A-:B-) and the chromosome. In these participants six different STs were detected, namely ST23, $ST23_{SLV}$, ST34, ST58, ST86 and ST131.

Prolonged carriage of ESBL in humans

Out of 134 participants with at least three analysed samples, five pig farmers from five different farms carried an ESBL producing *E. coli* more than once. Three out of these five pig farmers carried the same ESBL gene ($bla_{CTX\text{-}M\text{-}1}$) more than once. In two out of these three pig farmers (farm 1 and farm 4), isolates involved belonged to identical STs and encoded $bla_{CTX\text{-}M\text{-}1}$ on identical plasmid

Table 2a. Plasmid (sub)types and *E. coli* sequence types of human and pig isolates within the same farm.

		Sampling moment											
		0 months*			6 months			12 months			18 months		
	Origin	Gene	Plasmid	ST	Gene	Plasmid	ST	Gene	Plasmid	ST	Gene	Plasmid	ST
Farm 1	Human				CTX-M-1 CTX-M-1	IncI1 7/7 IncI1 7/7	48 (1) 1670 (1)	CTX-M-1	IncI1 3/3	10 (1)	CTX-M-1 CTX-M-1 CTX-M-1	IncI1 3/3 IncI1 7/7 IncI1 7/7	1486 (1) 48 (1) 1670 (1)
	Pigs				CTX-M-1 CTX-M-1 CTX-M-1	IncI1 3/3 IncI1 3/3 IncI1 3/3	58 (3) 398 (1) 410 (1)	CTX-M-1 CTX-M-1 CTX-M-1	IncI1 3/3 IncI1 3/3 IncI1 38/3	58 (1) 398 (1) 58 (1)	CTX-M-1 CTX-M-1 CTX-M-1 CTX-M-1 CTX-M-1	IncI1 3/3 IncI1 3/3 IncI1 7/7 IncI1 7/7 IncI1 38/3	1486 (1) 1952 (1) 58 (1) 398 (1) 1486 (1)
Farm 4	Human a	CTX-M-1	IncI1 3/3	453 (1)	CTX-M-1	IncI1 3/3	453 (1)						
	Human b	CTX-M-1	IncI1 3/3	453 (1)									
	Pigs	CTX-M-1 CTX-M-1 CTX-M-1	IncI1 3/3 IncI1 7/7 IncI1 7/7	453 (3) 58 (1) 453 (1)	CTX-M-1 CTX-M-1	IncI1 3/3 IncI1 38/3	453 (1) 453 (4)						
Farm 5	Human				CTX-M-1	IncI1 7/7	48 (2)				CTX-M-14	F2:A-:B-	10 (1)
	Pigs				CTX-M-1 CTX-M-1	IncI1 3/3 IncI1 278$_{SLV}$/-	58 (2) 1607 (1)				CTX-M-14 CTX-M-14 CTX-M-14	F2:A-:B- F2:A-:B- F2:A-:B-	10 (1) 2064 (1) 227 (1)
Farm 9	Human	CTX-M-1	IncI1 7/7	540 (1)	CTX-M-15	F-:A20:B1	12 (1)						
	Pigs	CTX-M-1 CTX-M-1 CTX-M-1 CTX-M-1	IncI1 7/7 IncI1 7/7 IncI1 7/7 IncI1 7/7	540 (2) 101 (1) 6593 (1) 10$_{SLV}$ (1)	CTX-M-15	Chromosome	12 (1)						
Farm 10	Human							CTX-M-1 CTX-M-1	IncI1 7/7 IncI1 7/7	6593 (1) 6404 (1)			
	Pigs							CTX-M-1 CTX-M-1 CTX-M-1	IncI1 7/7 IncI1 7/7 IncI1 7/7	6404 (1) 6587 (1) 101 (3)			

		Sampling moment											
		0 months			6 months			12 months			18 months		
	Origin	Gene	Plasmid	ST	Gene	Plasmid	ST	Gene	Plasmid	ST	Gene	Plasmid	ST
Farm 16	Human				CTX-M-1	IncI1 7/7	910 (1)						
	Pigs				CTX-M-1	IncI1 7/7	328 (1)						
					CTX-M-1	IncI1 7/7	638 (1)						
					CTX-M-1	IncI1 7/7	4040 (1)						
					CTX-M-1	IncI1 7/7	6859_{SLV}						
					CTX-M-1	IncI1 7/7	7618_{SLV}						
Farm 18	Human a	TEM-52	X1	10 (2)									
	Human b							TEM-52	X1	302 (1)			
	Pigs	TEM-52	X1	10 (1)									
		TEM-52	X1	101 (1)				TEM-52 var	X1	302 (1)			
		TEM-52	X1	154 (1)				TEM-52 var	X1	10 (3)			
		TEM-52	X1	877 (1)				TEM-52 var	X1	5579 (1)			
		TEM-52	X1	5579 (1)									
Farm 19	Human a	CTX-M-1	IncI1 7/7	711 (1)				CTX-M-1	IncI1 7/7	227 (2)			
	Human b	CTX-M-1	IncI1 7/7	101 (1)									
	Pigs	CTX-M-1	IncI1 7/7	711 (1)				CTX-M-1	IncI1 7/7	227 (1)			
		CTX-M-1	IncI1 7/7	10 (1)				CTX-M-1	IncI1 7/7	88 (1)			
		CTX-M-1	IncI1 7/7	227 (2)				CTX-M-1	IncI1 7/7	218 (1)			
		CTX-M-1	IncI1 7/7	3321 (1)				CTX-M-1	IncI1 7/7	711 (2)			
Farm 22	Human	CTX-M-14	F2:A-:B-	48 (1)									
		CTX-M-14	ColE2	3079 (1)									
	Pigs	CTX-M-14	F2:A-:B-	744 (2)									
		CTX-M-14	F2:A-:B-	3595 (1)									
		CTX-M-14	F2:A-:B13	48 (1)									
		CTX-M-14	F2:A-:B42	2946_{SLV} (1)									

* Plasmid subtype and MLST was constructed from WGS in one human and two pig isolates from farm 4 and one human and one pig isolate from farm 20 during the first sampling moment [3].

Table 2b. Plasmid subtypes and *E. coli* sequence types of human isolates obtained on farms without ESBL harboring pig isolates.

	Sampling moment	Gene	Plasmid	ST
Human (farm 11)	6 months	CTX-M-15	Chromosome	131 (1)
Human (farm 14)	6 months	CTX-M-1	IncI1 58/58	34 (1)
		CTX-M-1	IncI1 58/58	23 (1)
Human (farm 15)	6 months	CTX-M-2	IncI1 12/12	58 (1)
Human (farm 17)	12 months	CTX-M-1	IncI1 3/3	23_{SLV} (1)
Human (farm 23)	0 months	CTX-M-1	F2:A-:B-	86 (1)
		CTX-M-1	IncI1 3/3	58 (1)
		CTX-M-1	IncI1 Untypable*	86 (1)

*Exact matches found only for 4 out of 5 loci included in the plasmid MLST scheme [repI1 (2) - ardA (1) – trbA (4) -pilL(2)], while no positive PCR was obtained for the sogS gene after repeated attempts. The partial allelic profile obtained could correspond to pST-3/pCC-3, pST-101 or pST-220 based on the IncI1 plasmid MLST database (https://pubmlst.org/bigsdb?db=pubmlst_plasmid_seqdef).

subtypes in different sampling moments (Table 2a). For one pig farmer (farm 19), only the $bla_{CTX\text{-}M\text{-}1}$ carrying plasmid subtype was identical over time. For the two remaining farmers, different genes, strains and plasmid subtypes were observed over time. The genetic characteristics of ESBL-producing *E. coli* from humans and their pigs are listed in Table 2a.

Association between ESBL-producing *E. coli* in humans and pigs

Out of the total of 146 human participants, 134 provided a faecal sample during at least three sampling moments. Therefore, 22 out of 542 human observations were excluded from further analysis, which were all negative for ESBL. Characteristics of human participants are listed in Table 3.

From the total of 17 human ESBL carriers, 12 were living and working on nine farms, where ESBL-positive isolates from pigs were collected as well within the same sampling moment (18 human ESBL-positive observations). The remaining five human ESBL carriers were living on five farms where ESBL was not detected in pigs. Human ESBL carriage was univariately associated to the presence of ESBL in pigs (OR=8.9, 95% CI=2.9-27.4) and the average number of hours working on the pig farm per week (OR=1.04, 95% CI=1.01-1.06). Of the confounders considered, age and gender were significantly associated univariately (OR=1.04, 95% CI=1.01-1.07 and OR=5.3, 95% CI=1.4-19.9 respectively). Both age and gender were moderately correlated to average number of hours working on the pig farm per week (ρ=0.49 and ρ=0.54 respectively).

After mutual adjustment in bivariate analyses the association between human ESBL carriage and average number of working hours on the pig farm per week remained, while the association with age and gender became statistically non-significant. To avoid over-adjustment in multivariate analysis, only average number of working hours on the pig farm per week was considered for multivariate analysis in addition to the presence of ESBL in pigs. Results of univariate analysis are presented in Table 4.

In multivariate analysis human ESBL carriage was associated to both presence of ESBL in pigs (OR=8.7, 95% CI=3.5-25.6) and average number of hours working on the pig farm per week (OR=1.04, 95% CI=1.02-1.06). The final model is presented in Table 4. When restricting the final model to isolates harbouring $bla_{CTX\text{-}M\text{-}1}$ (16 positive observations from 12 participants) both associations remained (presence of $bla_{CTX\text{-}M\text{-}1}$ in pigs (OR=14.0, 95% CI=3.5-55.6) and average number of hours working on the pig farm per week (OR=1.02, 95% CI=1.00-1.05)).

Table 3. Baseline characteristics of participants who obtained at least three samples (n=132)*.

Human characteristics	Frequency (%)
Gender	
Male	77 (58)
Female	55 (42)
Category	
Farmer	45 (34)
Family of farmer	70 (53)
Employee	17 (13)
Age	132 (mean 36, range 7-79)
Age <18 years	30 (23)
Age 18-65 years	99 (75)
Age >65 years	3 (2)
Average number of hours working on the farm per week	126 (mean 25, range 0-80)
0	39 (31)
1-20	26 (21)
≥20	61 (48)
Smoking	
Yes	11 (8)
No	121 (92)

* Measured at the start of the study period (first sampling moment). Differences in characteristics between the first and further sampling moments were minor. Two out of 134 participants didn't provide a sample at the first sampling moment.

Table 4. Longitudinal univariate and multivariate analyses for ESBL carriage in pig farmers, family members and employees.

Determinant	No* or mean	Univariate OR (CI)	Multivariate OR (CI)
Presence of ESBL in pigs			
Yes	159	8.9 (2.9-27.4)	8.7 (3.0-25.6)
No	362	Ref.	Ref.
Average number of hours working on pig farm per week (per hour)	24 ± 24	1.04 (1.01-1.06)	1.04 (1.01-1.06)
Per 10 hours		1.4 (1.1-1.7)	1.4 (1.2-1.7)
Age (per year)	36 ± 17	1.04 (1.01-1.07)	
Per 10 years		1.45 (1.05-1.99)	
Gender			
Male	302	5.3 (1.4-19.9)	
Female	219	Ref.	
Smoking			
Yes	37	1.5 (0.2-12.9)	
No	473	Ref.	

* Based on total number of observations. Ref = reference category

Discussion

The number of farms with ESBL-producing *E. coli* present in pigs declined over time as well as the prevalence in humans. Human prevalence changed from 6% in the beginning to 2% at the end, which is roughly comparable to the prevalence of 5% reported in the general population in the Netherlands[11,12]. However, 12 out of the 17 (71%) participants positive for the presence of ESBL-producing *E. coli* carried the $bla_{CTX\text{-}M\text{-}1}$ gene at least once. In contrast, a low carriage of $bla_{CTX\text{-}M\text{-}1}$ gene among the general population in the Netherlands is observed. In previous Dutch studies among 1695 residents of Amsterdam, 2432 residents living in the vicinity of livestock farms and 4177 residents of the Netherlands, prevalence of $bla_{CTX\text{-}M\text{-}1}$ gene carriage ranged from 12 till 18%[11-13]. In addition, these studies revealed that the human related $bla_{CTX\text{-}M\text{-}15}$ gene was the most frequent gene among the Dutch general population, whereas in our study this gene was found in only two participants, underscoring that pig farmers and the general population differ on their ESBL gene type carriage[14].

In most cases, the ESBL gene type found in pig farmers was similar to the predominantly or exclusively detected ESBL gene type in pig isolates on the

same farm (Table 1), suggesting transmission between farmers and their animals. Considering that $bla_{CTX\text{-}M\text{-}1}$ is frequently found in livestock[1,4,15,16], transmission is likely to occur between animals and humans. However, the co-existence of $bla_{CTX\text{-}M\text{-}15}$ in both a human and pooled pig sample within one farm, suggests that transmission could be bi-directional. On seven farms a similar combination of gene, plasmid (sub)type and strain ST was observed in human and pig isolates within the same sampling moment, suggesting clonal transmission which was confirmed on isolates from farm 4 in a previous study[3]. On four farms a similar plasmid (sub)type without an identical ST was observed, indicating the possibility of horizontal gene transfer as well.

The epidemiological association between the presence of ESBL-producing *E. coli* in humans and pigs was already shown cross-sectionally[2] and confirmed by the longitudinal analysis described here. Both presence of ESBL in pigs and the duration of exposure were relevant for human ESBL carriage. Considering their daily intensive contact with livestock, clonal transmission is likely to occur between animals and animal caretakers. This is supported by the epidemiological association found in this study, combined with the genetic similarities of ESBL-producing *E. coli* obtained in humans and pigs within the same farm. While at the same time a great diversity in plasmid (sub)type and strain ST of ESBL-producing *E. coli* isolates from both humans and pigs was observed between farms.

Only two pig farmers of 134 people living and/or working on a pig farm carried identical isolates over time. For one of these pig farmers (farm 4), the isolates found in pigs were identical over time as well. Persistent carriage or repeated transmission events due to ongoing exposure to ESBL in pigs can both be an explanation for these observations. Duration of ESBL carriage can be dependent on strain factors. In a Dutch study in the general population, isolates harboring $bla_{CTX\text{-}M\text{-}1}$ were lost more easily than other gene types[8]. In a health care setting, ESBL-producing *E. coli* clone ST131 was more persistent than other ESBL-producing *E. coli*[17]. Several of the STs from human origin detected on farms where ESBL was present in pigs (ST10, ST48, ST227, ST302, ST453, ST540, ST711, ST1486, ST1670) has been previously associated with *E. coli* from porcine origin, whereas they have incidentally been described in *E. coli* from humans[18-20]. Within-pig farm epidemiology of ESBL-producing *E. coli* is mostly facilitated by animal related STs with potentially low capability of colonization and maintenance in human enteric cavity, leading to low prevalence and low percentage of persistence among pig farmers in spite of their close contact with their pigs.

This study provides genetic characteristics of ESBL-producing *E. coli* in pigs and pig farmers repeatedly over time. Great diversity was seen at the level of strain, gene and plasmid in human and pig derived isolates within and between farms and over time, which highlights the complex and dynamic epidemiology of ESBL-producing *E. coli* within pig farms. We documented both clonal dissemination and horizontal gene

transfer of ESBL genes between pigs and pig farmers. The possibility of transmission was confirmed by the epidemiological associations between ESBL carriage in humans and the presence of ESBL-producing *E. coli* in pigs on the farm and duration of contact with pigs using longitudinal data. Prolonged carriage of ESBL in pig farmers was observed incidentally.

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UND en VARKENSSLAGERU

Chapter 6

ESBL carriage in pig slaughterhouse workers is associated with occupational exposure

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Abstract

We investigated the prevalence of extended-spectrum beta-lactamase (ESBL) carriage in slaughterhouse workers and the association with occupational exposure to slaughter animals and products. Stool samples from 334 employees in a Dutch pig slaughterhouse were obtained. Presence of ESBL was determined by selective plating, microarray analysis and gene sequencing. Questionnaires were used to collect personal and occupational information. The overall prevalence of ESBL carriage was 4.8% (16/334). All ESBL-producing isolates were *Escherichia coli*. The ESBL genes detected were $bla_{\text{CTX-M-1}}$ (n=8), $bla_{\text{CTX-M-15}}$ (n=3), $bla_{\text{CTX-M-27}}$ (n=2), $bla_{\text{CTX-M-24}}$ (n=1), $bla_{\text{CTX-M-55}}$ (n=1), and $bla_{\text{SHV-12}}$ (n=1). A higher prevalence of ESBL was seen in workers in jobs with as tasks 'removal of lungs, heart, liver, tongue' (33%) and 'removal of head and spinal cord' (25%). For further analysis, participants were divided in two groups based on potential exposure to ESBL as related to their job title. One group with an assumed higher exposure to ESBL (e.g. stable work, stabbing, dehairing, removal of organs) and another group with an assumed lower exposure to ESBL (e.g. refrigeration, packaging, expedition). In the 'higher exposure' group, ten out of 95 (10.5%) were carrying ESBL versus six out of 233 (2.6%) in the 'lower exposure' group. Human ESBL carriage was significantly associated with job exposure in the slaughterhouse (Odds Ratio=4.5, 95% CI=1.6-12.6). Results suggest that ESBL carriage in slaughterhouse workers overall is comparable to the Dutch population. Within the slaughterhouse population a difference in carriage exists depending on their position along the slaughter line and tasks involved.

Introduction

In humans, infections with extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae are associated with increased burden of disease and costs[1]. Livestock can carry ESBL-producing Enterobacteriaceae with $bla_{CTX\text{-}M\text{-}1}$ as the most prevalent ESBL gene found in Europe[2]. ESBL-producing Enterobacteriaceae can be transferred from animals to humans through food or direct contact[3,4]. Direct contact with livestock mainly occurs in an occupational setting. In farmers, carriage of ESBL-producing Enterobacteriaceae is associated with the presence of ESBL-producing Enterobacteriaceae in animals[3,5,6]. Slaughterhouse workers might also be occupationally exposed to ESBL-producing Enterobacteriaceae. Depending on the job task, slaughterhouse workers have frequent contact with live animals, animal carcasses or animal products. ESBL-producing Enterobacteriaceae have frequently been found in intestinal content of pigs at the slaughter level[7-9]. Besides that, ESBL-producing Enterobacteriaceae can also be detected on pig carcasses at the slaughterhouse[10]. A higher prevalence of antimicrobial resistant *Escherichia coli* in pig slaughterhouse workers has been reported[11-13]. However, carriage of ESBL-producing Enterobacteriaceae in slaughterhouse workers has not been established yet. In addition, *E. coli* contamination on carcasses seems to be reduced during the slaughter process[14,15]. Therefore exposure, and as a consequence prevalence of carriage, of ESBL-producing Enterobacteriaceae might be dependent on the working area and job task within a slaughterhouse. We investigated the prevalence of ESBL carriage in pig slaughterhouse workers and the association with occupational exposure.

Materials and Methods

Study design

A large pig slaughterhouse in the southern part of the Netherlands (Vion, Boxtel) was visited by the researchers during one week in June 2015. All slaughterhouse workers with a slaughter process related job were asked to participate in the study by the use of flyers and information on screens. In total, 1781 eligible slaughterhouse workers were employed by the slaughterhouse at the time of the study. Participants were asked to provide a faecal specimen and to fill out a consent form and a questionnaire containing items on personal and occupational information, including job function in the slaughterhouse. Due to the heterogeneity in nationality of the slaughterhouse workers (14 different nationalities), the flyer, questionnaire and consent form were provided in 11 different languages. Participants were motivated for participation with a voucher worth of 25 euro. Faeces samples and documents were handed in

to the researchers at the slaughterhouse within the same week. At the same day, for the purpose of detecting ESBL-producing Enterobacteriaceae, <1 gram faeces was collected from the tube by the use of a swab and stored in the refrigerator immediately. All swabs were sent refrigerated in one batch to the laboratory. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. The Medical Ethical Committee of the University Medical Centre Utrecht confirmed that the Medical Research Involving Human Subjects Act did not apply for this study and that therefore an official approval by the Medical Ethical Committee was not required (protocol no. 14-346/C). All participants gave written informed consent.

Laboratory analysis

At the day of arrival in the laboratory, all faecal specimen were analysed for the presence of ESBL-producing Enterobacteriaceae by selective plating. Samples were suspended in 10 ml LB-medium with cefotaxime (1 ug/ml) and incubated overnight at 37°C. Approximately 10 µL of each suspension was streaked on MacConkey agar plates with cefotaxime (1 µg/ml) and incubated overnight at 37°C. Individual colonies with different morphology were selected for bacterial species identification performed by MALDI-TOF MS. All isolates suspected of producing ESBLs were selected for further molecular analysis to confirm the presence of ESBL genes. DNA was isolated using DNeasy 96 Blood & Tissue Kit (Qiagen, Hilden, Germany). A β-lactamase microarray assay (Check-MDR CT101, Checkpoints, Wageningen, the Netherlands) was used to detect genes encoding carbapenemases (KPC and NDM), ESBLs (CTX-M groups 1, 2, 8/25 and 9, TEM , SHV), and AmpCs (CMY-1 /MOX, ACC, DHA, ACT/MIR, CMY-2, FOX). DNA from ESBL/AmpC microarray positive isolates was amplified and sequenced with group-specific primers to determine the exact gene type[16]. DNA sequences were interpreted with Basic Local Alignment Search Tool (National Center for Biotechnology Information). Plasmids encoding the ESBL (or AmpC) genes were determined using a transformation-based approach. Purified plasmid DNA was electro-transformed into *E. coli* DH10B cells (Invitrogen, Van Allen Way, CA USA) under the following conditions: 1.25 kV/cm, 200 Ω, 25 µFar[17]. When strains recovered from workers were not giving transformants (workers 12 and 15), conjugation was performed using the plasmid-free rifampin-resistant *E. coli* E3110 as a recipient strain for liquid-mating assays in 1:1 ratio as previously described[17]. Transformants were selected on LB agar supplemented with cefotaxime (1 ug/ml), whereas transconjugants on LB agar with rifampin (100 ug/ml) and cefotaxime (1 ug/ml). PCR-based replicon typing (PBRT) on the transformants and multilocus sequence typing (MLST) on the parental strains were performed to analyse genetic similarities of isolates and plasmid content[17].

Table 1. Distribution of slaughterhouse workers and ESBL carriage over different job tasks.

		Frequency		
	Job task	**Total**	**ESBL carriers**	**Non-ESBL carriers**
'higher exposure' group	Stable / lairage part, stunning part	12	0	12
	Stabbing and bleeding part	5	0	5
	Dehairing and hanging part	6	1	5
	Removal of penis, stab wound and loosing/ligation anus (anal duct)	6	0	6
	Evisceration	10	1	9
	Removal of lungs, heart, liver, tongue	12	4	8
	Inspection platform	8	0	8
	Dressing (first, second), removal abdominal fat, diaphragm	17	1	16
	Removal of head, spinal cord	8	2	6
	Organs (tongues, hearts, livers, kidneys)	11	1	10
'lower exposure' group	Refrigeration and cooling area	26	0	26
	Cutting room	75	2	73
	Deboning area	63	1	62
	Packaging	30	2	28
	Expedition (moving and shipment)	8	0	8
	Other (e.g. technical services)	31	1	30

Statistical analysis

Slaughterhouse workers were classified as ESBL positive if any ESBL gene was detected in their faeces sample. Statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). The association between carriage of ESBL-producing Enterobacteriaceae in humans and job function was calculated with logistic regression analysis (proc GENMOD). For this analysis, participants were divided in two groups based on potential exposure within their specific job task. One group with an assumed higher exposure to ESBL-producing Enterobacteriaceae consisted of job tasks in the early stages of the slaughter process (e.g. stable work, stabbing, dehairing, removal of organs i.e. direct contact with pigs and pig carcasses). The other group, assumed to have lower exposure to ESBL-producing Enterobacteriaceae, consisted of job tasks in the latter stages of the slaughter process (e.g. refrigeration, packaging, expedition i.e. contact with meat or meat products) (see Table 1). When participants reported to have more than one job function, they were assigned to the first job function at the slaughter line. Other potential risk factors as nationality, consumption of raw meat, hospitalization, use of antimicrobials, living on a farm, traveling and potential confounders age, gender, and smoking were analysed univariately as well and selected for multivariate analysis

when p-value was below 0.2. Model selection was performed by a backward procedure. Model fit was checked with the QIC-statistic (Quasi-likelihood under the Independence model Criterion). The final model retained variables significant at p≤0.05.

Results

Human stool samples from 334 employees were obtained. Most of the participants were male and did not originate from the Netherlands (Table 2). The overall prevalence of ESBL carriage was 4,8% (16/334), and all ESBL-producing isolates were *E. coli*. The ESBL genes detected were $bla_{CTX\text{-}M\text{-}1}$ (n=8), $bla_{CTX\text{-}M\text{-}15}$ (n=3), $bla_{CTX\text{-}M\text{-}27}$ (n=2), $bla_{CTX\text{-}M\text{-}24}$ (n=1), $bla_{CTX\text{-}M\text{-}55}$ (n=1), and $bla_{SHV\text{-}12}$ (n=1). In addition, three other slaughterhouse workers carried an AmpC gene [$bla_{CMY\text{-}2}$ (n=2) and $bla_{DHA\text{-}1}$ (n=1)]. Plasmids encoding ESBL genes were assigned to multi-F (n=6), I1α (n=4), N (n=3), and K (n=1) replicon types. For two isolates, transformation and conjugation experiments failed to give transformants and transconjugants suggesting the chromosomal location of these genes. MLST revealed that ESBL-producing *E. coli* belonged to 11 different sequence types (STs), with ST131 (n=4), ST88 (n=3) and ST93 (n=3) as the most frequent ones. All molecular aspects are listed in Table 3.

ESBL carriage was not equally distributed across different jobs in the slaughterhouse. A higher prevalence of ESBL seemed to appear in workers involved in 'removal of lungs, heart, liver, tongue' (33%) and 'removal of head and spinal cord' (25%) (See Table 1). In the 'higher exposure' group, ten out of 95 (10,5%) were carrying ESBL versus six out of 233 (2,6%) in the 'lower exposure' group. Human ESBL carriage was significantly associated with job exposure in the slaughterhouse (Odds Ratio (OR)=4.45, 95% CI=1.57-12.62). Smoking was univariately and negatively borderline significantly associated with ESBL carriage (OR=0.31, 95% CI=0.09-1.12) as well as traveling (OR=0.36, 95% CI=0.13-1.04). None of the other potential risk factors (nationality, consumption of raw meat, hospitalization, use of antimicrobials, living on a farm) or confounders (age, gender) were significantly associated with ESBL carriage. In multivariate analysis, the effects of smoking and traveling appeared independent of job exposure (only a slight change in OR) and did not remain in the final model. Therefore the final model only contained job exposure as a risk factor for ESBL carriage.

When looking at ESBL carriage by nationality, Romanian workers had a higher prevalence [five out of 41 (12,2%)] compared to the other three well represented nationalities: Hungarian [one out of 46 (2,2%)], Dutch [3 out of 60 (5,0%)], and Polish [five out of 119 (4,2%)]. However, 23 out of 41 (56,1%) of the Romanians worked in the 'higher exposure' group, which is a considerably higher percentage than within Hungarian, Dutch, and Polish slaughterhouse workers [seven out of 46 (15,2%), nine out of 60 (15,0%), and

30 out of 119 (25,2%) respectively]. When in the final model the effect of job exposure was adjusted for Romanian nationality, the association between ESBL carriage and job exposure hardly changed (OR=4.85, 95% CI=1.54-15.24). No significant interaction between Romanian nationality and job exposure was observed for ESBL carriage.

Table 2. Overview of participant characteristics and Odds Ratios from the univariate analysis for the probability of ESBL carriage.

	Frequency			
Characteristics of participants	**Total**	**ESBL carriers**	**Non-ESBL carriers**	**OR (95% CI)**
Exposure group in slaughter process (n=328)				
'higher exposure' group	95	10	85	4.45 (1,57-12,62)
'lower exposure' group	233	6	227	Ref.
Age (mean) (n=328)	39.8	38.1 (n=16)	39.9 (n=312)	0,99 (0,94-1,03)[1]
Gender (n=324)				
Male	276	14	262	2,51 (0,32-19,54)
Female	48	1	47	Ref.
Nationality (n=305)				
Non-Dutch[2]	245	12	233	0,98 (0,27-3,58)
Dutch	60	3	57	Ref.
Consumption of raw meat (n=294)				
Yes	106	4	102	0,70 (0,21-2,28)
No	188	10	178	Ref.
Hospitalization in the past 12 months (n=295)				
Yes	32	2	30	1,28 (0,28-5,96)
No	263	13	250	Ref.
Use of antimicrobials in the past 12 months (n=323)				
Yes	48	3	45	1,34 (0,37-4,90)
No	275	13	262	Ref.
Living on a farm (n=297)				
Yes	17	0	17	NE
No	280	13	267	
Traveling in the past 12 months (n=311)				
Yes	216	7	209	0,36 (0,13-1,04)
No	95	8	87	Ref.
Smoking (n=321)				
Yes	140	3	137	0,31 (0,09-1,12)
No	181	12	169	Ref.

Ref. = Reference category; NE = Not estimated due to empty cells.

[1] Per one year increase.

[2] Not from the Netherlands: Cape Verdean (n=1), Ghanese (n=1), Hungarian (n=46), Latvian (n=3), Polish (n=119), Portugese (n=12), Romanian (n=41), Slovakian (n=20), Turkish (n=2).

Focusing on $bla_{CTX\text{-}M\text{-}1}$ gene solely, the prevalence was 7.4% (7/95) in the 'higher exposure' group and 0.4% (1/233) in the other group. When analysis was restricted for $bla_{CTX\text{-}M\text{-}1}$ alone, the association between ESBL carriage and exposure group was confirmed (OR=18.46, 95% CI=2.24-152.16).

From the 74 participants who reported to have more than one job title, 11 participants mentioned job titles not within the same exposure group. This misclassification of exposure might have changed the association between exposure and ESBL carriage. When a sensitivity analysis was performed by switching these 11 participants from the 'higher exposure' to the 'lower exposure' group, the observed association between ESBL carriage and occupational exposure did not change significantly and had a similar point estimate (OR=5.36, 95% CI=1.89-15.25).

Table 3. Molecular aspects of ESBL/AmpC positive *E. coli* isolates from slaughterhouse workers.

Slaughterhouse worker	ESBL/AmpC gene	Plasmid rep/ inc-type	Sequence types (ST)	Job task
1	$bla_{CTX\text{-}M\text{-}1}$	IncI1α	88	Evisceration
2	$bla_{CTX\text{-}M\text{-}1}$	IncI1α	88	Removal of lungs, heart, liver, tongue
3	$bla_{CTX\text{-}M\text{-}1}$	IncI1α	88	Removal of lungs, heart, liver, tongue
4	$bla_{CTX\text{-}M\text{-}1}$	IncI1α	10	Dressing (first, second), removal abdominal fat, diaphragm
5	$bla_{CTX\text{-}M\text{-}1}$	IncN	93	Removal of head, spinal cord
6	$bla_{CTX\text{-}M\text{-}1}$	IncN	93	Removal of head, spinal cord
7	$bla_{CTX\text{-}M\text{-}1}$	IncN	93	Organs (tongues, hearts, livers, kidneys)
8	$bla_{CTX\text{-}M\text{-}1}$	IncFIA-FII	131	Packaging
9	$bla_{CTX\text{-}M\text{-}15}$	IncK	156	Cutting room
10	$bla_{CTX\text{-}M\text{-}15}$	incFIB-FII	410	Deboning area
11	$bla_{CTX\text{-}M\text{-}15}$	IncFIA-FII	131	Dehairing and hanging part
12	$bla_{CTX\text{-}M\text{-}24}$	-[1]	354	Packaging
13	$bla_{CTX\text{-}M\text{-}27}$	IncFIA-FIB-FII	131	Other (e.g. technical services)
14	$bla_{CTX\text{-}M\text{-}27}$	IncFIB-FII	131	Cutting room
15	$bla_{CTX\text{-}M\text{-}55}$	-	95	Removal of lungs, heart, liver, tongue
16	$bla_{SHV\text{-}12}$	IncFIB-FIC-FII	665	Removal of lungs, heart, liver, tongue
17	$bla_{CMY\text{-}2}$	IncI1α	752	Cutting room
18	$bla_{CMY\text{-}2}$	IncI1α	752	Cutting room
19	$bla_{DHA\text{-}1}$	IncFIA-FII	38	Stabbing and bleeding part

[1] No successful transformation or conjugation.

Discussion

The study revealed that slaughterhouse workers were more likely to carry ESBL when working in the early slaughtering steps (before chilling of the pig carcasses) than slaughterhouse workers working from this slaughter step forward i.e. working in the cooling, cutting and deboning area. Overall prevalence of ESBL carriage was 4.8%, which is more or less comparable to numbers found in two recent Dutch studies in residents of Amsterdam (8.6%) and residents living in the vicinity of livestock farms (4.5%)[18,19]. However, the ESBL prevalence differed between the assumed higher and lower exposed groups (10.5 vs 2.6%) pointing at higher occupational exposures during specific tasks performed by individual slaughterhouse workers.

A previous study in the same slaughterhouse showed a decline in presence and counts of *Salmonella* on skin surface and the exterior of carcasses from bleeding till chilling[20]. In addition, a decline in presence of *E. coli* during the slaughter process, especially after chilling of the pig carcasses, has been described in literature[14,15,21,22]. It is likely that ESBL-producing *E. coli* shows the same decline as *E. coli* in general, although we can only assume since exposure to ESBL was not measured in this study. This could explain the higher carriage of ESBL among slaughterhouse workers when involved in the slaughter steps before chilling. For some slaughter steps only low numbers of participants could be included. As a consequence, it was not possible to estimate differences in ESBL carriage in more detail. However, when focusing on the descriptive figures, ESBL carriage is considerably higher when working in the slaughter steps 'removal of lungs, heart, liver, tongue' and 'removal of head and spinal cord'. Exposure to ESBL might be higher for workers in these slaughter steps, because both steps involve handling of the throat area including the pharyngeal tonsils which are colonized with high counts of several bacteria (potentially including ESBL-producing *E. coli*)[23,24].

In this study, $bla_{\text{CTX-M-1}}$ was the most predominantly detected ESBL gene in pig slaughterhouse workers. Out of the ESBL positive slaughterhouse workers, eight were carrying $bla_{\text{CTX-M-1}}$ (50%), which is significantly higher than the proportions of $bla_{\text{CTX-M-1}}$ reported in two Dutch studies. In ESBL positive residents of Amsterdam and ESBL-positive residents living in the vicinity of livestock farms 26 out of 145 (18%) and 13 out of 99 (13%) carried a $bla_{\text{CTX-M-1}}$ gene respectively[18,19]. In addition, $bla_{\text{CTX-M-1}}$ is also the most frequently found ESBL gene in slaughter pigs in the Netherlands[25]. Moreover, pig farmers were found to carry the same ESBL gene type as exclusively or predominantly detected in their pigs, of which $bla_{\text{CTX-M-1}}$ was most frequently found in both of them[3]. In contrast, $bla_{\text{CTX-M-15}}$ has been found mostly in humans in a clinical setting and in the Dutch human population, but not that often in livestock[2,18,26]. A great diversity was seen in STs, therefore clonal transmission between slaughterhouse workers is

probably not the dominant route of transmission. Besides, half of the STs (ST10, ST38, ST88, ST93, ST354) have been previously associated with *E. coli* from pig origin[8,27,28]. In addition, IncN and IncI1 plasmids are known to play a role in the dissemination of $bla_{CTX\text{-}M\text{-}1}$ in *E. coli* in livestock, including pigs[29-31]. Although no samples from carcasses or slaughter products were obtained, these results regarding ESBL gene types, plasmids and STs are suggesting transmission from animals to humans.

Besides occupational exposure, none of the analysed determinants (nationality, consumption of raw meat, hospitalization, use of antimicrobials, living on a farm, traveling, age, gender, and smoking) were found to be risk factors for ESBL carriage in slaughterhouse workers. ESBL carriage was higher in slaughterhouse workers with a Romanian nationality compared to other nationalities, although the association between ESBL carriage and occupational exposure remained when adjusting for Romanian nationality. Besides that, the effect of being in the 'higher exposure' group is similar for Romanian slaughterhouse workers as for the other nationalities, considering the non-significant interaction. Also, in three out of five Romanian ESBL carriers the ESBL gene type detected was $bla_{CTX\text{-}M\text{-}1}$. Smoking and traveling were protective factors for ESBL carriage univariately, although they did not retain in the final model. From the 173 non-Dutch slaughterhouse workers who reported their traveling destiny, 88% travelled to their country of origin. Although traveling frequencies were not assessed, traveling might be interpreted as long distance commuting. To our knowledge, there is no explanation described in literature for any effect of smoking on ESBL carriage.

Although ESBL carriage was detected in slaughterhouse workers, the risk of acquiring ESBL-producing *E. coli* when handling meat intensively seems to be limited. When this is extrapolated to consumers, exposure to ESBL in pork through handling might not be of a high public health concern. In combination with environmental exposure data, this information can be used in formal risk assessments. Although not addressed in this study design, duration and persistence of ESBL carriage can vary greatly[32,33]. Duration of ESBL carriage is not only important for assessing the risk of infection, but for the risk of transmission of ESBLs from slaughterhouse workers into the general population as well.

The results suggest that the overall carriage in slaughterhouse workers is comparable to the Dutch population, but the proportion of the $bla_{CTX\text{-}M\text{-}1}$ gene (commonly found in livestock) is higher. Differences exist between slaughterhouse workers depending on their job tasks. A higher prevalence of ESBL carriage was found in slaughterhouse workers working before the chilling process of the carcasses compared to workers in the cooling area, cutting and deboning departments.

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UND en VARKENSSLAGERU

Chapter 7

General discussion

ESBL in humans exposed to pigs or pig products

Prevalence of ESBL in humans exposed to pigs or pig products

Extended-spectrum beta-lactamases (ESBL) were detected in *Escherichia coli* (*E. coli*) isolates from 17 participants out of the total of 146 pig farmers, family members and employees. Overall human prevalence of ESBL carriage in people living and/or working with pigs or pig slaughter products ranged from 2-6% depending on study population and sampling moment, which is comparable to the general population in the Netherlands[1-3]. However, ESBL prevalence was higher when including only humans with regular occupational exposure to ESBL carrying pigs (24% over all sampling moments) or pig slaughter products at the early slaughtering steps with potential higher environmental exposure (i.e. before chilling of the pig carcasses) (10,5%). Contrarily, prevalence of ESBL carriage was low in the subgroups without exposure to ESBL carrying pigs or pig slaughter products at the end of the slaughter line (i.e. cutting and deboning area).

A healthy worker selection effect might have biased the comparisons between the populations under study and the general population. The populations under study were in general healthy, with a low prevalence of comorbidities that may also be associated with a higher use of antimicrobials. Previously described risk factors for human ESBL carriage are the use of antimicrobials, hospitalization and traveling to high risk countries[2,4]. These determinants were hardly observed among the study participants, which likely contributed to the low baseline prevalence found.

The role of occupational exposure to pigs or pig products

Exposure to ESBL has been determined on farms. ESBL was present in pigs on 28-45% of the farms, depending on the sampling moment. In addition, ESBL (CTX-M1 group) was detected in settled airborne dust on 26% of the farms at least once during the study period. ESBL carriage in humans was associated to both presence of ESBL in dust as presence of ESBL in pigs. Next to direct contact with ESBL carrying pigs, airborne transmission might be of importance. Airborne dust levels in pig farms are high, and inhalation and subsequent ingestion of ESBL containing dust can lead to a substantial ESBL dose when working for prolonged periods with animals. In addition, the duration of exposure (i.e. average time spent working on the farm) was of influence on the odds of being an ESBL carrier. These observations indicate that transmission of ESBL from pigs to humans is very likely. The risk of transfer of ESBL between livestock and farm workers have been described by others as well[5-9].

In slaughterhouse workers, ESBL carriage was higher at the beginning of the slaughter line (i.e. dirty slaughter tasks) compared to the end of the line (i.e. cleaner slaughter tasks). This finding is suggestive for transmission of ESBL from pig slaughter products to humans. A decline in level of contamination with ESBL-producing *E. coli*

on carcasses and slaughter products was assumed during the slaughter process, as described for generic *E. coli* in literature[10-13].

In general, it can be stated that within the study groups a division can be made in high exposure vs low/no exposure (contact vs no contact with ESBL carrying pigs or ESBL contaminated products) corresponding with higher and lower prevalence of ESBL carriage respectively. Also, within the farming community, 70% of the humans who carried ESBL at least once were exposed to ESBL carrying pigs on a daily basis. In other words, a vast majority of the ESBL carriers were pig farmers on farms where ESBL positive pigs were present.

Genetic characteristics of ESBL-producing *E. coli* in humans and pigs

The epidemiological evidence for transmission is supported by molecular relatedness of ESBL harboring isolates from humans and pigs obtained from the same farm. The distribution of the different gene types found on farms was similar in human and pig isolates. On pig farms, $bla_{\text{CTX-M-1}}$ was the most detected ESBL gene in humans (12 out of 17 participants) and pig isolates (67%), followed by $bla_{\text{TEM-52}}$ and $bla_{\text{CTX-M-14}}$. Half of the *E. coli* isolates obtained from slaughterhouse workers also harbored a $bla_{\text{CTX-M-1}}$. These gene types, especially $bla_{\text{CTX-M-1}}$ have been detected in animal and human isolates derived from pig farms, while $bla_{\text{CTX-M-15}}$ is mostly observed in the general population[1-3,5,7,14,15]. In addition, human and pig isolates within the same farm harbored exclusively or predominantly similar ESBL gene types.

On farms where ESBL-producing *E. coli* was detected in both pigs and humans, identical combinations of gene, plasmid (sub)type and sequence type (ST) were frequently detected in pig and human derived isolates. The similarity in gene, plasmid (sub)type and ST of human and pig isolates obtained from the same farm is suggestive for clonal transmission from pigs to humans or vice versa. The occurrence of clonal transmission was confirmed on one farm by whole genome sequencing results by de Been et al.. Only six single nucleotide polymorphisms were found between a human $bla_{\text{CTX-M-1}}$ harboring isolate and two $bla_{\text{CTX-M-1}}$ harboring isolates obtained from pigs on the same farm, which is comparable to similarity seen between isolates within an outbreak[16].

Several STs found in pig farmers and pig slaughterhouse workers have been associated with *E. coli* previously detected in livestock, including pigs[17-19]. Additionally, IncI1 plasmids were observed most frequently and described to be involved in the dissemination of $bla_{\text{CTX-M-1}}$ in *E. coli* in pigs[20-22]. A great diversity of plasmids and STs within and between farms and over time was observed, especially in the pig population. With a sampling interval of 6 months, it is unlikely that the same animals were sampled twice. Only two farmers carried an isolate that was identical in ESBL gene, plasmid (sub)type and *E. coli* ST repeatedly.

Transmission of ESBL from the farming community to the general population

Direct transmission of ESBL from farmers and livestock to the general population

People with frequent (occupational) contact with ESBL carrying livestock are at risk of acquiring an animal derived ESBL[5-9,23]. However, further transmission from these humans to the general population is probably of more limited importance. No observations pointing at within household transmission were observed in our study. First, no family members of ESBL carriers were determined to carry an ESBL, without having daily occupational exposure to ESBL positive pigs themselves. On two farms, a family member was carrying an ESBL next to the farmer, but both ESBL carriers had daily contact with ESBL positive pigs. On one of these farms, ST differed between the two ESBL carriers present. Second, if human to human transmission would have occurred, we would have expected a higher overall human ESBL prevalence than observed. To our knowledge, potential transmission from farmers to their household members have only been suggested in two cases on broiler farms[8,24].

Contrarily, within household transmission has been described frequently in non-farming populations. In a Dutch study quantifying within-household transmission from (previously) hospitalized patients colonized with ESBL-producing Enterobacteriaceae to their household members, a transmission probability of 67% was modeled[25]. Several other studies showed that carriage of ESBL-producing Enterobacteriaceae in household members of ESBL colonized patients was more prevalent (17-27%) than observed in the general population[26-30]. Amongst 168 household members from people who had acquired an ESBL during travel, 13 acquired an ESBL of the same ESBL group as the index traveler after return. The probability of ESBL transmission from ESBL positive travelers to a household member was 12%[4]. Seven out of 49 family members of 22 families with an ESBL positive adopted child carried an ESBL. Within household transmission of ESBL was demonstrated in five cases within four families based on molecular relatedness[31]. Co-carriage of ESBL in child-parent pairs from 1016 families in the Netherlands occurred more frequently than expected based on pure chance, suggesting within household transmission[32,33].

The frequency, intensity and nature of contact within households with a colonized patient probably differs from a farming community, since patients are likely to get care from their family members (caregiving household members). This might explain a part of the difference in within-household transmission found between the farming community and other study populations. Next to that, human acquired ESBL-producing Enterobacteriaceae (hospital, community or traveling

abroad) might be more efficient in colonizing humans than ESBL-producing *E. coli* from an animal origin. Also, duration of ESBL carriage might be different between the farming community and the general population, based on differences in strain factors and exposure behavior of carriers. For instance, CTX-M group 9 is associated with longer carriage than CTX-M group 1. Isolates harboring $bla_{\text{CTX-M-15}}$ persist longer than isolates harboring $bla_{\text{CTX-M-1}}$, and colonization with ESBL-producing *E. coli* clone ST131 lasts longer than colonization with other ESBL-producing *E. coli*[4,34-36]. *E. coli* strains such as ST131 are often reported in humans and known to carry $bla_{\text{CTX-M-15}}$ frequently[37]. In addition to strain factors, the use of antimicrobials and several clinical characteristics such as urine catheters and chronic wound have been associated with prolonged colonization in patients[38,39]. A longer period of colonization enhances the probability of transmission. Longitudinal ESBL data from farming communities is extremely sparse. Based on our study, persistent carriage does not occur often in farmers. The hypothesized shorter period of colonization could be another partial explanation for the low human to human transmission within the farming community. This immediately also raises the question whether ESBL carriage in the farming community is persistent or a matter of transient carriage.

Livestock is considered to be a reservoir for ESBL-producing Enterobacteriaceae. Amongst all family members who had no animal contact, but did live on a farm with ESBL positive pigs, no ESBL carriers were detected. This makes the probability of transmission without entering stables nihil. This finding was endorsed in a large study in residents living in close proximity to farms, where living closely to livestock and farm(s) was not detected as a risk factor. Out of 2432 participants living in a livestock-dense area in The Netherlands, 92 humans carried an ESBL gene (3,7%) of which only 13 carried $bla_{\text{CTX-M-1}}$ (0,5%)[1]. In 2009, potential exposure to resistance genes through air exposure was studied in the context of an explorative study on health effects for humans living near livestock farms. Amongst other measurements, PM10 samples were obtained by active air sampling on five different locations in a livestock dense area, next to a control location in an urban area[40]. Except for the urban control location, at all five locations, the presence of $bla_{\text{CTX-M}}$ group 1 in outside air was determined. No quantitative results could be obtained, since DNA levels were low and measured just above the limit of detection (personal communication Dr. Inge Wouters, October 2019). Although emission of ESBL from livestock farms is thus shown to be likely; overall results do not indicate that potential exposure to ESBL in a livestock dense area leads to human ESBL carriage.

In general, exposure to ESBL in farmers and/or livestock is considered to be low for the general population. Especially relatively to other potential ESBL sources. If within household transmission in the farming community is not common and

no higher prevalence is seen in residents in a livestock-dense area, the attribution of this reservoir to ESBL in the general population is expected to be low. In a recently performed source attribution analysis on ESBL prevalence in the Netherlands, the relative attribution of human reservoirs was considered to be dominant. Most community-acquired ESBL/AmpC producing *E. coli* was attributed to human to human transmission in the open community (60.1%). Non-occupational contact with livestock and secondary transmission from farmers was modeled to be responsible for 3,6% and 1,0% of the carriage of ESBL/AmpC producing *E. coli* in the open community[41]. Their study highlighted the importance of human to human transmission, despite the several reservoirs and risk factors involved and the multifactorial nature of ESBL transmission dynamics. Regardless the relatively small attribution fraction, monitoring and intervention for non-human sources is still needed, since spillover of ESBL from these reservoirs to the general population does occur.

Molecular relatedness between farming communities, livestock and the general population

In a meta-analysis where genetic characteristics of ESBL isolates from different ESBL reservoirs were compared, one of the least similar gene distributions to the human general and clinical populations were those of farming communities. A close linkage of plasmid replicon types originated from livestock and humans was not demonstrated. In a broader sense, most livestock or food associated reservoirs did not show a high level of genetic similarity with human isolates obtained in the general population or a clinical setting[42]. A high level of similarity in ESBL genes was seen in isolates from farmers and their livestock, which can be explained by their epidemiological linkage. Whole-genome sequencing confirmed the likelihood of clonal transmission of *E. coli* between pigs and pig farmers, but failed to determine clonal transmission of isolates from poultry meat to humans. However, a prominent role of mobile genetic elements in the transmission of ESBL from food producing animals to humans was suggested by the predominance of identical ESBL-carrying IncI-plasmids in cephalosporin-resistant *E. coli* derived from human and animal sources[16]. In the United Kingdom a large genomic surveillance of *E. coli* isolates from human bloodstream infections, livestock and meat was performed. Core genome analysis determined genetically distinct livestock and patient isolates. In addition, analysis of mobile genetic elements identified a low prevalence of shared antimicrobial resistance genes (including $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-15}}$), between livestock and humans[43].

Overall, there is limited data to assess potential in depth genetic similarity between ESBL-producing *E. coli* detected in the general population and ESBL-producing *E. coli*

derived from livestock. However, similarity of ESBL-producing *E. coli* originating from humans and livestock is likely within farming communities. Community (or hospital) acquired carriage of ESBL harboring isolates genetically similar to livestock originated isolates is far less evident without the presence of such an epidemiologic cluster. The exact role of vertical transfer of ESBL from animals to humans remains unclear due to the complex epidemiology of plasmid transfer, the lack of data including all potential ESBL sources and conflicting results.

Transmission of ESBL from livestock to the general population through food

Transmission of ESBL from food producing animals to humans via the food chain has been suggested, mostly based on partial molecular relatedness, i.e. parallel occurrence of strains, ESBL genes or plasmids, and/or bacterial clones[44-46]. Recent studies observed a low level of similarity in genetic characteristics and gene distributions between ESBL-producing *E. coli* originated from humans and meat as already stated earlier[16,42,43]. In the above described source attribution analysis on ESBL prevalence, retail meat (chicken, bovine, turkey, pork, and sheep/goat) accounted for 11,2% of community acquired ESBL/pAmpC *E. coli* carriage in the Netherlands[41]. In a quantitative microbial risk assessment exposure of ESBL-producing *E. coli* has been assessed by using several parameters, such as ESBL prevalence and concentration on meat, reduction by processing, and food consumption, either retracted from literature or field experts. It was estimated that human total exposure to ESBL/pAmpC producing *E.coli* through consumption of contaminated meat is generally low[47]. No clear evidence for the contribution of meat consumption to human ESBL carriage can be retracted from studies in vegetarians. A vegetarian diet has been described as protective factor for acquiring an ESBL, however, mostly in humans traveling to high risk countries[48]. Another study presented no significant difference in prevalence of colonization with ESBL-producing Enterobacteriaceae between vegetarians and meat consumers[49].

Next to meat consumption, preparation of meat in the kitchen has been described to play a role in the transmission of ESBL from livestock to humans[50,51]. In our study in slaughterhouse workers, prevalence of ESBL in people working in the later steps of the slaughter line was low compared to the general population. The risk of acquiring ESBL-producing *E. coli* when handling pig meat intensively over working day periods seems to be limited. As a result, exposure to ESBL in pork for consumers through handling might not be of a high public health concern. However, this is likely depending on the contamination of food products, kitchen hygiene and consumption behavior. To our knowledge, no observational data is available on the relative attribution of handling meat by consumers to the prevalence of ESBL in the general population.

Overall it remains unclear in what precise extent exposure to ESBL-producing Enterobacteriaceae through meat attributes to the presence of ESBL in humans. However, exposure to ESBL originated from livestock is likely to be higher through the food chain than via direct contact given the low frequency of the latter for an average citizen of the Netherlands.

The relative attribution of livestock and animal derived food products to ESBL carriage in the general population might be different in the non-Western society due to differences in societal factors, food hygiene, farming practices such as backyard farming and living circumstances. The distinction between the farming community and the general population is probably less stringent since humans and animals are potentially living more closely together. Therefore, transmission dynamics of ESBL from livestock to the general population can be hypothesized to be different in non-Western communities.

The potential of prevention and reduction of ESBL in humans related to livestock

Reduction of human exposure to ESBL in livestock

Since livestock is a reservoir for ESBL and appointed to be of (at least any) relevance to the maintenance of ESBL in the general population, interventions to reduce or prevent ESBL in animals are of common interest. Several management measures focusing on internal and external biosecurity have been considered to have an influence on the presence of ESBL in livestock, but clear evidence is very limited[14,52]. In our study, the use of a hygiene lock as only entrance to the farm was a protective factor, which is a common biosecurity measure taken on farms. The presence of goats on the farm was determined as a risk factor for the presence of ESBL in pigs, which was hypothesized to be a proxy for poorer management practices since a causal relationship is not that plausible. Antibiotic stewardship has been appointed as the intervention with the highest potential to reduce ESBL in livestock. In countries with a high use of antimicrobials a high prevalence of ESBL in livestock and farm workers is seen[53,54]. There is a known association between the use of cephalosporins and the emergence of ESBL[55,56]. In our study, incidental cephalosporin use was associated to the presence of ESBL in pigs. Also, ESBL-*E. coli* carriage in pigs significantly decreased during the study period. A Danish study showed a significantly higher frequency of ESBL-producing *E. coli* on farms with high consumption of third- or fourth-generation cephalosporins compared to farms with no use of third- or fourth-generation cephalosporins[7].

When reduction of ESBL in livestock on farms is possible by several measures, the number of transmission events of ESBL from animals to humans in an occupational setting might decline. Gut colonization at hospital admission is associated to subsequent clinical infections with ESBL[57-60]. Therefore, reducing ESBL carriage could be beneficial to the personal health of farmers, although the link between fecal carriage of ESBL and clinical manifestations in this healthy worker population is not observed particularly or determined yet.

By reducing ESBL in livestock, ESBL exposure to humans via farmers, livestock and the food chain might be decreased which would reduce potential public health risks. However, the potential of reduction of antimicrobial use in livestock as prevention for the carriage of antimicrobial resistance in the general population has not been carefully investigated. Within the Netherlands the use of antimicrobials in livestock dropped drastically since 2011 due to demands by the Dutch government and actions taken by livestock farmers and veterinarians. Moreover, the use of third- and fourth generation cephalosporins is very limited since some farm sectors introduced private initiatives in 2011 and legal limitations for veterinary prescriptions were set by the government in 2013[61]. Despite this immense decline in the use of antimicrobials in livestock in general and cephalosporins specifically, no decline in clinical infections with ESBL in the general population is seen. Based on the national antimicrobial resistance surveillance system, the percentage of ESBL in human clinical isolates of Enterobacteriaceae in the Netherlands was estimated to have increased from 2% to 6% over the past five years[62]. Regardless the pitfalls in antimicrobial stewardship in livestock as preventive measure to reduce the burden of ESBL in the general population, the reduced use of antimicrobials might attribute to preventing new emerging antimicrobial resistance in livestock. In addition, a healthy use of antimicrobials might be key to prevent an increase of ESBL presence in livestock and transmission to humans.

Prevention of transmission of ESBL from livestock to humans

Next to reducing the exposure to ESBL in livestock, intervening in ESBL carriage could be possible by prevention of the actual transfer from livestock to humans. By using farm practices focusing on personal preventive measures, farmers might reduce transmission of ESBL from animals through direct contact or airborne transmission. Although no information on specific actions regarding ESBL-producing Enterobacteriaceae on farms is available, it could be hypothesized that measures focusing on preventing transmission of Enterobacteriaceae and infection control in general would work as well. Applying hand hygiene by washing and using gloves adequately could contribute to a reduced transmission by inhibiting the fecal-oral transmission route[63-65].

As stated earlier, transmission of ESBL through the food chain is probably more likely than via direct contact with livestock. Therefore, consumers can reduce the risk of acquiring ESBL in the kitchen during food preparation by applying proper hand hygiene, prevent cross-contamination and cooking products adequately[51,66]. In addition, interventions at slaughter level could be considered. A decline in presence of (ESBL-producing) *E. coli* on carcasses during the slaughter process has been described[10,12,67]. This suggests a potential for lowering ESBL contamination of the end product. Whether these preventive measures will reduce the prevalence of ESBL in humans can only be hypothesized at this moment.

The complexity of ESBL epidemiology

Diversity in genetic characteristics of ESBL-producing *E. coli*

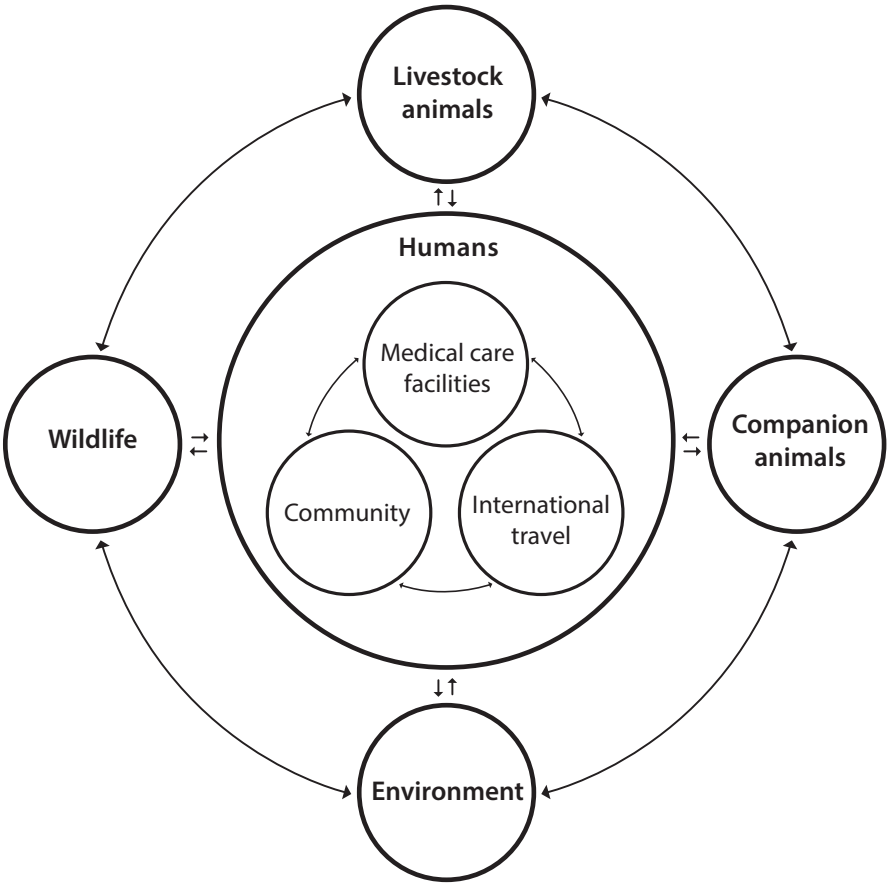
Epidemiology of ESBL is complex and creates challenges due to a great number of aspects. First, a great diversity and variety in genetic characteristics of genes, mobile elements and bacterial hosts exists. Differences in these genetic characteristics are seen within and between reservoirs and drive the different dynamics for the gene, plasmid, strain combinations. Certain genes and hosts prevail over others, which can be different depending on region of the world. Molecular relatedness is therefore essential to assess the likelihood of transmission. Second, transfer of ESBL genes can occur both by clonal transmission or by horizontal gene transfer. Molecular relatedness between mobile elements in ESBL harboring isolates from different reservoirs is described with or without a direct epidemiologic link (such as isolates collected within a farming community or during an outbreak)[6,9,16,37]. Evidence of clonal transmission is available from outbreak data in a hospital setting, but also commonly suggested within households and on farms[8,16,32,68]. Third, as mentioned before, great variation is seen in duration of colonization with ESBL, which can also differ depending on gene and strain type involved and the source of origin[4,34-36]. Due to the combination of gene, plasmid, strain and host, carriage can be prolonged, but also transient carriage versus colonization is discussed. To add another layer, mutations and shifts in prevalence and occurrence of certain types of genes, plasmid and strains can happen over time.

Dynamics of ESBL between reservoirs

As mentioned several times before in this thesis, many reservoirs for ESBL are known. However, the dynamics within and between the different reservoirs is rather complex as visualized in figure 1. Exchange of ESBL occurs frequently within and between certain human reservoirs including the community and patients in several care

facilities[25,26,69]. Thereby, traveling to certain high risk countries, hospitalization and the use of antimicrobials as risk factors of acquiring ESBL are contributing to the maintenance of ESBL in humans[2,4,70,71]. Human reservoirs are also connected to several animal and environmental ESBL harboring reservoirs such as livestock (poultry, veal calves, pigs, dairy cows) and food, companion animals, wildlife, recreational water, sewage and soil[45,46,72-77]. Different routes of transmission involved are direct contact, the food chain, and through the environment. Considering the overlap of genetic profiles of ESBL harboring strains, there is no doubt about the connectivity between all reservoirs. However, next to transmission a parallel but separate increase of ESBL probably has occurred over time as well. This can be explained by dynamics within reservoirs and similar selection pressure by the use of antimicrobials in several reservoirs.

Figure 1. ESBL transmission routes and reservoirs (based on Ewers et al.[15])



The effect of sampling in ESBL epidemiology

In order to gain insight in the dynamics of ESBL within and between reservoirs, data by observations need to be collected. Given the diversity in ESBL characteristics, prevalence, relevant transmission types and routes and changes over time and per region in the different reservoirs, selection by sampling might influence the interpretation of results in case of ESBL epidemiology. A priori expectations are often difficult to set. Therefore, it is a challenge to present a complete overview of the dynamics of ESBL. By taking several smaller snapshots, important information can be missed or misinterpreted regarding the bigger picture. For example, for estimating the relative attribution and relevance of ESBL in livestock for human ESBL carriage occupational groups are often used. These groups form only a small portion of the general population and are not representative in terms of exposure. Heterogeneity of data introduces a challenge for pooled or meta-analysis as well. Selection often occurs on several levels, such as animal species, farm, animals, samples, isolates and time period. These selection steps all might introduce bias, which can lead to misinterpretation of the data. Molecular relatedness within or between reservoirs can be left undiscovered when the diversity in strains, genes and plasmids is high.

However, with an ongoing screening and collection of data more knowledge will be gained, which can be used to estimate sample size and selection on several levels more accurately. Large longitudinal studies with repeated measurements and molecular techniques will be needed to monitor ESBL in the different reservoirs and improve understanding of dynamics.

The use of mathematical modeling in ESBL epidemiology

Recently, mathematical modelling has been used increasingly for a better understanding of ESBL dynamics[4,25,36,41,50,78]. The estimation of the attribution of the different ESBL reservoirs to carriage of ESBL in the general population is difficult given all complex aspects of ESBL dynamics. The presence of ESBL in livestock and livestock related reservoirs has been determined quite extensively in the past decade in the Netherlands[8,73,79,80]. Therefore some model and parameter estimates can be derived from observational studies. The high data density improves the precision of estimates. These models can at least provide qualitative insights in the transmission dynamics of ESBL. However, the limitations of these models are not always made explicit and for proper interpretation it remains essential to carefully check potential biases and uncertainties of underlying data used and assumptions made. Thus, in many cases quantitative estimates may not always be sufficiently accurate and precise. Quantitative data regarding the microbial load is often not available, which is essential information to be able to predict exposure from a reservoir with sufficient precision. In addition, information on dose-response relations is not available. Also,

the relevance of different transmission routes within and between reservoirs still needs to be determined. Therefore, assumptions have to be made to define the exact burden of ESBL related to livestock and the potential to reduce this burden. Moreover, as mentioned above, sampling selection at different levels with all the known varieties of combinations available is of influence on data results and therefore the outcome of models. A lower level of robustness of data introduces insecurities. Alterations in data driven parameters and estimated numbers can lead to changes in terms of quantitative outcomes. On a global scale, even more assumptions have to be made, since most data on ESBL is collected in Western society and results cannot be extrapolated to other parts of the world with different living circumstances. Therefore, an ongoing collection of (longitudinal) data is needed in a variety of circumstances.

Conclusions

ESBL-producing Enterobacteriaceae are widely present in livestock. Farm workers are, due to the high intensity and frequency of contact with livestock, of a higher risk of carrying an animal derived ESBL than humans in the general population. However, considering the low occurrence of within household transmission within the farming community and the low prevalence of ESBL in residents living in a livestock dense area, further transmission from farm workers to the general population is not very likely or at least expected to be relatively low. The relative attribution of livestock reservoirs to ESBL in the community is considered to be low. Preventing and reducing ESBL in livestock to lower the risk of transfer from animals to humans is likely to be possible for care takers, but to our knowledge no evidence for successful decreasing prevalence of ESBL in the general population by focusing on livestock reservoirs is available. Considering the complexity of ESBL epidemiology, the variety of reservoirs and transmission routes involved, and the alterations in dynamics over time, a *One Health* perspective is required. Focusing on a solely ESBL reservoir might lower the (already low) relative attribution but may have a minor effect on the total ESBL prevalence in the general population. Prevention should be addressed from multiple perspectives and should include initiatives and close collaboration by all stakeholders involved. It is still hard to produce a quantitative estimate of the potential decrease in burden of disease caused by ESBL by reducing the presence of ESBL in the different known sources. The complexity of ESBL dynamics within and between all the potential ESBL reservoirs urges the need for a *One Health* approach.

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UND en VARKENSSLAGERU

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Summary

Introduction

Extended spectrum beta-lactamases (ESBL) are an emerging concern in public health. The major ESBL-producing Enterobacteriaceae involved in human infections are *Escherichia coli* (*E. coli*) and *Klebsiella pneumonia*. ESBL can cause resistance to different beta lactam antimicrobials, including penicillins and cephalosporins. Most regularly observed ESBL genes belong to the TEM, SHV, and CTX-M groups. Occurrence of CTX-M group, specifically $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-14}}$, has increasingly been reported in humans over the past decade. The genes encoding for ESBL are often located on plasmids, which can be transferred within and between different bacterial species. In humans, infections with ESBL producing Enterobacteriaceae are associated with increased burden of disease and costs.

In addition to ESBL in humans, a variety of ESBL have been identified in bacteria derived from the environment and animals. In Europe $bla_{\text{CTX-M-1}}$ is common in poultry, cattle and pigs. High use of antimicrobials and inappropriate use of cephalosporins in livestock production are considered to be associated with the emergence and high prevalence of ESBL in animals. Transmission of ESBL from livestock to humans may occur through direct contact with livestock. This thesis focused on the presence of ESBL in pigs, the pig farming community and pig slaughterhouse workers. Risk factors for ESBL carriage in humans, both farmers and slaughterhouse workers, were determined next to risk factors for the occurrence of ESBL in pigs at farm level. Genetic characteristics of ESBL-producing *E. coli* detected in humans and pigs were compared within and between farms, and over time.

Data collection

On 40 pig farms, ESBL genes were detected longitudinally in pigs, humans and dust. In addition, sequence type and plasmid (sub)type of ESBL-producing *E. coli* were determined for a selection of isolates. Personal and farm characteristics were collected using questionnaires. At a Dutch pig slaughterhouse ESBL genes, plasmid and strain information were determined in *E. coli* isolates from slaughterhouse workers distributed over different occupational tasks.

Main results

ESBL genes were detected in *E. coli* isolates in 17 out of 146 humans, in pigs on 18 out of 39 farms and in dust on ten out of 38 farms at least once during the longitudinal study on pig farms. In 16 out of 334 pig slaughterhouse workers ESBL genes were detected in *E. coli* isolates. However, presence of ESBL genes was higher when focusing on potential high risk groups, such as farmers and slaughterhouse workers

with an assumed higher exposure to ESBL (e.g. stable work, stabbing, dehairing, removal of organs).

Among people working/living on pig farms, frequent contact with pigs carrying ESBL was associated with ESBL carriage. Working hours on the farm (indicating direct contact with pigs) and presence of ESBL in pigs on the farm were associated with human ESBL carriage. In addition to direct contact with pigs, the possibility of airborne transmission of ESBL genes (CTX-M group 1) was suggested by the association between human ESBL carriage and the presence of ESBL genes in dust.

In pig slaughterhouse workers, ESBL carriage was significantly associated with job exposure. Slaughterhouse workers were more likely to carry an ESBL when working at the beginning of the slaughter process (before chilling of the pig carcasses) than slaughterhouse workers working from this slaughter step forward, i.e. working in the cooling, cutting and deboning area.

The presence of ESBL in pigs declined over time, both in number of farms where pigs carrying ESBL were present as in number of ESBL positive pig fecal samples detected on the farms. The observed steady reduction in total AMU, resulting from national policies, did not explain these changes. However, the incidental use of cephalosporins was associated to the presence of ESBL carrying pigs on farms. Additional farm management practices focused on improved biosecurity (e.g. presence of a hygiene lock, pest control delivered by a professional) were associated with a lower probability of presence of ESBL in pigs on farms. The restriction in the use of cephalosporins has likely resulted in a decrease of ESBL carriage on pig farms. Therefore, reduction of ESBL in pigs seems a possibility. This might lead to reduced transmission of ESBL from pigs to humans, which could be beneficial for humans occupationally exposed to pigs.

In both farmers and slaughterhouse workers as well as in pigs, $bla_{\text{CTX-M-1}}$ was the most frequently detected ESBL gene. Human and pig isolates within the same farm harbored identical ESBL gene types. On several farms, identical combinations of ESBL genes, sequence types and plasmid (sub)types were detected in *E. coli* isolates from humans and pigs, which is suggestive for clonal transmission. In addition, similarity was observed in gene and plasmid subtype, leaving open the possibility of horizontal gene transfer. Overall, a great variety in plasmid (sub)types and *E. coli* sequence type (ST) was seen. Five pig farmers carried an ESBL repeatedly, of which two carried an identical combination of gene, plasmid subtype and ST over time. Although transmission of ESBL from pigs to pig farmers did occur, prolonged carriership was observed incidentally.

Human to human transmission of ESBL between farmers and their family members probably did not occur. On two farms, a family member was carrying an ESBL next to the farmer. On both farms, all ESBL carriers had daily contact with ESBL positive pigs and on one farm ST differed between the two ESBL carriers present. In pig slaughterhouse workers, substantial diversity was seen in ST, therefore clonal transmission between slaughterhouse workers is probably not the dominant route of transmission.

Concluding remarks

This thesis provides prevalence data and genetic characteristics of ESBL-producing *E. coli* in pig slaughterhouse workers, pig farmers and pigs. Great diversity was seen at the level of strain, gene and plasmid in human and pig derived isolates within and between farms and over time, which highlights the complex and dynamic epidemiology of ESBL-producing *E. coli* within pig farms. Considering their daily intensive contact with livestock, clonal transmission is likely to occur between pigs and pig farmers. This is supported by the epidemiological association between ESBL carriage in humans and duration of contact with pigs using longitudinal data, combined with the genetic similarities of ESBL-producing *E. coli* obtained in humans and pigs within the same farm. Prolonged carriage of ESBL in pig farmers was observed incidentally. ESBL carriage in slaughterhouse workers was associated with high risk occupational tasks.

Pig farmers and pig slaughterhouse workers are, due to the high intensity and frequency of contact with pigs or pig products, at risk of carrying animal derived ESBL. However, further transmission from these occupationally exposed groups to the general population is not very likely or at least expected to be relatively limited. Considering the complexity of ESBL epidemiology and the variety of transmission routes and reservoirs, a *One Health* approach is required for future ESBL research.

Samenvatting

Introductie

Extended Spectrum Bèta-Lactamase (ESBL) vormende bacteriën zijn in toenemende mate een risico voor de gezondheid van de mens. *Escherichia coli* (*E. coli*) en *Klebsiella pneumonia* zijn de voornaamste ESBL-producerende bacteriën betrokken bij infecties in mensen. ESBLs zijn enzymen die bèta-lactam antibiotica ineffectief kunnen maken. Daardoor zijn ESBL-producerende bacteriën resistent voor bepaalde groepen antibiotica zoals penicillines en cefalosporines. ESBL genen worden ingedeeld in verschillende groepen, waarbij TEM, SHV en CTX-M het meest voorkomen. ESBL genen uit de CTX-M groep, met name $bla_{CTX\text{-}M\text{-}15}$ en $bla_{CTX\text{-}M\text{-}14}$, komen in toenemende mate voor bij mensen. ESBL genen zijn vaak gecodeerd op plasmiden, welke overdraagbaar zijn tussen bacteriën. Deze plasmiden bepalen de dynamiek van de ESBL epidemiologie in hoge mate. Infecties met ESBL-producerende bacteriën in mensen gaan gepaard met een toenemende ziektelast en zorgkosten.

Niet alleen in mensen, maar ook in dieren en het milieu is een verscheidenheid aan ESBL genen aangetroffen. In pluimvee, runderen en varkens komt $bla_{CTX\text{-}M\text{-}1}$ veel voor. Het gebruik van antibiotica en inadequate toepassing van cefalosporines in landbouwhuisdieren wordt geassocieerd met een toename en hoge prevalentie van ESBLs in dieren. Overdracht van ESBLs van landbouwhuisdieren naar mensen vindt mogelijk plaats door (werk gerelateerd) contact met landbouwhuisdieren.

Dit proefschrift beschrijft het voorkomen van ESBLs in varkens, mensen die wonen en/of werken op een varkenshouderij en slachthuismedewerkers in een varkensslachthuis. Risicofactoren voor ESBL dragerschap bij varkenshouders en slachthuismedewerkers werden onderzocht. Daarnaast is ook gekeken naar risicofactoren voor het voorkomen van ESBLs in varkens op varkenshouderijen. Ook zijn de genetische kenmerken van ESBL-producerende *E. coli* afkomstig van mensen en varkens met elkaar vergeleken.

Verzameling van gegevens

Het voorkomen van ESBL genen is herhaaldelijk bepaald in mensen, varkens en stof op 40 varkenshouderijen. Daarnaast is voor een selectie van isolaten het *E. coli* sequentie type en het plasmide (sub)type bepaald. Persoonlijke- en bedrijfskenmerken werden verzameld met behulp van vragenlijsten. In een Nederlands varkensslachthuis zijn het ESBL gen, sequentie type en plasmide type bepaald in *E. coli* isolaten van slachthuismedewerkers, verdeeld over verschillende werkzaamheden gedurende het slachtproces.

Belangrijkste resultaten

ESBL genen zijn één keer of herhaaldelijk gedetecteerd in *E. coli* isolaten van 17 van de in totaal 146 herhaaldelijk onderzochte mensen op varkenshouderijen. ESBL genen zijn aangetroffen in varkens op 18 van de 39 varkenshouderijen en in stof op 10 van de 38 varkenshouderijen (gedurende tenminste één meetmoment). Bij 16 van de 334 varkensslachthuismedewerkers zijn ESBL genen gedetecteerd in *E. coli* isolaten. Binnen potentiële hoog risicogroepen, zoals varkenshouders en slachthuismedewerkers die taken uitvoeren met een verwachte hogere blootstelling aan ESBLs (bijvoorbeeld werken met levende dieren, steken, ontharen en verwijderen van organen), werden hogere ESBL dragerschap-prevalenties gezien.

ESBL dragerschap bij mensen was geassocieerd met regelmatig contact met varkens die ESBLs bij zich dragen op varkenshouderijen. Het aantal uren dat gemiddeld per week gewerkt werd in de stallen, als maat voor de duur van direct contact met varkens, en de aanwezigheid van ESBL positieve varkens op het bedrijf waren beiden geassocieerd met ESBL dragerschap bij mensen. Daarnaast was humaan ESBL dragerschap geassocieerd met de aanwezigheid van ESBL genen in stofdeeltjes in de lucht. Hierdoor lijkt, naast direct contact met varkens, ook luchtblootstelling aan ESBLs een rol te spelen.

In varkensslachthuismedewerkers was ESBL dragerschap geassocieerd met de veronderstelde blootstelling aan ESBLs tijdens specifieke taken van het slachtproces. ESBL dragerschap was waarschijnlijker wanneer werkzaamheden aan het begin van de slachtlijn (voor het koelen van het karkas) werden uitgevoerd dan werkzaamheden later in het proces (vanaf het koelen), zoals snijden en uitbenen.

Het aantal bedrijven met ESBL dragende varkens is afgenomen gedurende de studieperiode evenals het aantal positieve monsters per bedrijf. De gelijktijdige afname in het antibioticagebruik op varkensbedrijven verklaarde de daling van het aantal positieve bedrijven en monsters niet. Incidenteel gebruik van cefalosporines was daarentegen wel geassocieerd met de aanwezigheid van ESBL positieve varkens op een bedrijf. Een aantal maatregelen, met name die gericht waren op het verbeteren van de biosecurity (bijvoorbeeld de aanwezigheid van een hygiënesluis en het uitbesteden van ongediertebestrijding aan een professioneel bedrijf), bleek negatief geassocieerd met de aanwezigheid van ESBL positieve varkens op het bedrijf. De in het kader van het generieke antibioticareductiebeleid opgelegde restricties voor het gebruik van cefalosporines heeft waarschijnlijk geleid tot een daling van het voorkomen van ESBLs bij varkens. Daarmee lijkt het verder terugdringen van ESBLs op varkensbedrijven ook mogelijk. Dit zal naar verwachting, op basis van de bevindingen in dit project, leiden tot een verminderde transmissie van ESBLs van varkens naar mensen, met name bij varkenshouders.

In zowel veehouders, slachthuismedewerkers als varkens, kwam van alle ESBL genen $bla_{CTX\text{-}M\text{-}1}$ het meest voor. Bij mensen en varkens van hetzelfde bedrijf werden identieke type ESBL genen gevonden. Op meerdere bedrijven waren *E. coli* isolaten afkomstig van mensen en varkens van hetzelfde bedrijf tevens identiek in sequentie type en plasmide (sub)type, waardoor klonale transmissie waarschijnlijk lijkt. Ook was op een aantal bedrijven alleen gen type en plasmide (sub)type gelijk, wat duidt op de mogelijkheid van horizontale plasmide overdracht. Er is een grote verscheidenheid aan *E. coli* sequentie types en plasmide (sub)types gezien op de verschillende varkenshouderijen. In totaal droegen vijf varkenshouders herhaaldelijk een ESBL-producerende *E. coli* bij zich, waarvan bij twee varkenshouders identieke combinaties van gen type, plasmide subtype en sequentie type werden geobserveerd. Transmissie van ESBLs van varkens naar varkenshouders heeft plaatsgevonden, langdurig dragerschap werd slechts incidenteel gezien.

Humane transmissie van ESBLs tussen varkenshouders en hun familieleden heeft waarschijnlijk niet plaatsgevonden. Op twee bedrijven was, naast de varkenshouder, ook een familielid drager van het ESBL gen. Echter, op beide bedrijven hadden alle ESBL dragers dagelijks contact met varkens op ESBL positieve bedrijven. Op één van deze bedrijven was het sequentie type van de *E. coli* isolaten van de varkenshouder en het familielid verschillend. In slachthuismedewerkers werd een grote diversiteit in *E. coli* sequentie types gezien, hierdoor lijkt klonale transmissie tussen de medewerkers geen dominante rol te spelen bij de overdracht van ESBL genen binnen deze populatie.

Conclusies

In dit proefschrift worden prevalentie en genetische kenmerken van ESBL-producerende *E. coli* in varkensslachthuismedewerkers, varkenshouders en varkens en de samenhang in dragerschap bij mensen en dieren beschreven. De variatie in ESBL genen, sequentie types en plasmide (sub)types gevonden in *E. coli* isolaten afkomstig van mensen en varkens was groot, zowel binnen en tussen bedrijven als over de tijd. Dit laat zien hoe complex en dynamisch de epidemiologie van ESBLs op varkenshouderijen is. Gezien het dagelijks intensieve contact met hun dieren, is klonale transmissie van ESBL-producerende *E. coli* tussen varkens en varkenshouders zeer waarschijnlijk. Dit wordt ondersteund door de aangetoonde epidemiologische associatie tussen humaan ESBL dragerschap en de mate van contact met ESBL positieve varkens, gecombineerd met de genetische overeenkomsten in ESBL-producerende *E. coli* van varkenshouders en varkens binnen hetzelfde bedrijf. Langdurig ESBL dragerschap werd slechts incidenteel gezien bij varkenshouders. De meerderheid van de varkenshouders was slechts op één moment drager en

negatief tijdens een volgend meetmoment (gemiddeld 6 maanden later). ESBL dragerschap bij slachthuismedewerkers was geassocieerd met de werkzaamheden in het slachthuis.

Varkenshouders en varkensslachthuismedewerkers hebben door de hoge frequentie van intensief contact met varkens of varkensproducten een verhoogd risico op dragerschap van ESBLs afkomstig van varkens. Verdere transmissie van ESBLs van deze beroepsgroepen naar de algemene bevolking is minder waarschijnlijk en vindt waarschijnlijk in zeer beperkte mate plaats. De epidemiologie van ESBLs is complex. Er zijn meerdere transmissieroutes en reservoirs van belang. Daarom is voor verder toekomstig ESBL onderzoek een *One Health* benadering nodig.

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Curriculum Vitae

Wietske Dohmen was born in Utrecht, The Netherlands on August 16, 1981. She attended secondary school at K.S.G. De Breul in Zeist, from which she graduated in 1999. During several years of exclusion from acceptance for Veterinary Medicine at Utrecht University due to the numerus fixus system, she worked at Utrecht University Library, obtained her propaedeutic degree in Dutch Language and Culture and studied Veterinary Medicine at Ghent University. Finally, after being selected at Utrecht University in 2003, she obtained her doctoral degree in Veterinary Medicine at Utrecht University in 2008. After that, she started working as a junior researcher in epidemiological research at the Department of Farm Animal Health at Utrecht University. In 2011, she started her PhD project, described in this thesis, at the Institute for Risk Assessment Sciences, Utrecht University. During her PhD, she obtained her Master's degree in Biomedical Sciences within the master's program Epidemiology with a specialization in Infectious Diseases. Since 2016, she has been involved in several educational tasks focusing on One Health, including development of courses and teaching. She has been working on the development and implementation of the new master's program One Health and was program coordinator while it was running for the first time. From 2020 onwards, she is working at the Institute for Risk Assessment Sciences as Assistant Professor One Health. She will start working on a research project regarding circular farming practices with an integrative focus on environmental emissions, animal diseases, animal welfare and public health. In addition, she will continue her educational activities in the master's program One Health. Wietske is living with her husband and three children in Vianen, The Netherlands.

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